Alpha-Cleavage of the Prion Protein and the Putative Function of the Prion Protein in Lymphocyte Homeostasis

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José Barros Oliveira Martins

aus Portugal

Promotionskommitee

Prof. Dr. med. Dr. sc. h.c. Adriano Aguzzi
Prof. Dr. sc. Annette Oxenius
Prof. Dr. rer. nat. Burkhard Becher

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Summary

Prion diseases are fatal neurodegenerative disorders that have the particularity of being caused by an infectious proteinaceous agent. The main player in this disease is a structural modification of the endogenous non-pathogenic prion protein (PrP^{C}), termed PrP^{Sc}. Though PrP^{C} is widely expressed in the body and highly conserved in mammals, its physiological function remains elusive. Moreover, *Prnp*$^{-/-}$ mice devoid of PrP have no strong evident phenotype, apart from being completely resistant to prion diseases. Interestingly, many PrP^{C} mutants with deletions around the α-cleavage site of PrP^{C} are highly toxic when expressed in transgenic mice and this toxicity is rescued by coexpression of wild-type PrP^{C}. One of the main mysteries in the prion field is the elucidation of the molecular mechanisms that drive not only the toxicity of PrP^{Sc}, but also the pathology caused by these PrP^{C} deletion mutants.

To understand the principles behind PrP-dependent toxicity, first a model was conceptualized, which aimed to integrate and unify the largest possible set of experimental evidences already available in the literature. The goal was to conceive a framework that congregates a putative function of PrP^{C}, as well as the pathological features of prion diseases and of prionopathies caused by PrP^{C} deletion mutants. From all possible models considered, the most parsimonic one established that α-cleavage of the PrP^{C} central domain is crucial for regulating PrP^{C} function and for explaining PrP-dependent toxicity. The model was also based on a main set of assumptions, which are that internalization of uncleaved PrP^{C} contributes to cell activation, and that miss-regulation of this process results in cell over-activation and consequent cell death. Therefore, I decided to study these two paradigms, which are the α-cleavage, and the toxicity due to the putative cell hyper-activation derived from PrP^{C} miss-regulation.

Concerning α-cleavage, the main grounds sustaining its importance are that both PrP^{Sc} and all toxic PrP^{C} deletion mutants have a strong impairment on α-cleavage. In this project I tried to identify the PrP^{C} domains neighbouring the α-cleavage site, which play a role in mediating this proteolysis. Assessment of the α-cleavage rate of a large number of PrP^{C} mutants designed in this study revealed that neither substitution of the amino-acid residues at the cleavage site, replacement or inversion of the charged residues, mutation of the palindromic region, nor exchange of the

hydrophobic amino-acids, had major effects on the degree of α-cleavage. Also, short deletions at the cleavage site had only a mild effect on PrP^C proteolysis. However, an increase in the size of the deletion resulted in a gradual decrease of α-cleavage rate, which was virtually blocked in the deletion mutant $PrP^{\Delta(106-119)}$. These results suggest that cleavage of PrP^C is largely sequence independent, which is an uncommon feature that can find parallelism with the proteolytic plasticity of the γ-secretase.

To investigate the second paradigm, that PrP^C is a modulator of cell activation, and that miss-regulation of its function can result in cell death, the impact of PrP^C on lymphocyte activation and positive and negative selection of lymphocytes was assessed. This study suggested that PrP^C is strongly up-regulated in activated T-cells, and that it is found at high quantities in activated lymphocytes, in cells undergoing positive selection, and in populations that traditionally have high reactivity to antigens, like regulatory T-cells and marginal zone B-cells. An alteration of the lymphocytic homeostasis in mice deficient of or overexpressing PrP^C was also observed. This alteration could be explained by a deletion of the transgenic cells with high levels of PrP^C during the negative selection of auto-reactive cells, and by an arrest in development due to anergy of *Prnp*$^{-/-}$ lymphocytes. In addition, it was shown that the costimulatory CD3 molecule is down-regulated in circulating T-cells from PrP^C-overexpressing mice, probably in order to re-establish the activation threshold, which might have been decreased due to the high presence of PrP^C. Finally, I showed that PrP^C overexpression partially rescues the impairment on development of CD4 T-cells in mice devoid of MHC class II, which is essential for providing an activatory signal to these cells.

Taken together, here I propose a unifying model for PrP function and PrP-dependent toxicity, assessed the main assumption, and studied the principles governing α-cleavage, which is the main mechanism of this model.

Zusammenfassung

Prionenkrankungen sind fatale neurodegenerative Erkrankungen, deren Besonderheit es ist, ein infektiöses Protein als Ursache zu haben. Der Hauptverursacher dieser Erkrankung ist eine strukturell missgefaltete Version des endogenen, nicht-pathogenen Prionenproteins (PrP^{C}), genannt PrP^{Sc}. Obwohl PrP^{C} praktisch im gesamten Organismus vorkommt und die Sequenz in Säugetieren stark konserviert ist, blieb die Frage der physiologischen Funktion bisher ungelöst. Überdies haben *Prnp*$^{-/-}$ Mäuse ohne das Prion-Protein keinen stark ausgeprägten Phänotyp - ausser dass sie komplett resistent gegen Prionen-Erkrankung sind. Interessanterweise sind viele PrP-Mutationen mit einer Deletion in der Nähe der α-Schnittstelle von PrP^{C} extrem toxisch, wenn sie in transgenen Mäusen exprimiert werden. Diese Toxizität wird aber bei gleichzeitiger Expression von PrP^{C} aufgehoben. Eines der grossen Mysterien im Bereich der Prionen bleibt die Aufklärung der molekularen Mechanismen, die der Toxizität von PrP^{Sc} wie auch der Deletionsmutanten von PrP^{C} zugrunde liegen.

Um die Prinzipien, die hinter der PrP-abhängigen Toxizität stehen, zu beleuchten, entwarf ich ein Modell, das möglichst viele bereits publizierter experimenteller Beweise integriert. Das Ziel war ein Model, der sowohl die mögliche Funktion von PrP^{C}, wie auch die pathologischen Charakteristika von übertragbarer Prionenerkrankungen und der Deletionsmutanten erklärt. Das plausibelste Model wäre, dass die α-Schnittstelle in der zentralen Domäne des Prion-Proteins essentiell für die Funktion des Proteins ist. Die Konsequenz wäre, dass eine Fehl-Regulation der proteolytischen Spaltung eine konstante Internalisation von aktivem PrP zur Folge hat, was eine Erhöhung des Aktivierungsstatus der Zelle hat und schlussendlich den Zelltod auslöst. Daraufhin beschloss ich, die Spaltung an der α-Schnittstelle und die Toxizität, die möglicherweise von der Hyperaktivierung, aufgrund einer PrP^{C}-Fehlregulation verursacht wird, zu untersuchen.

Dass die α-Spaltung tatsächlich äusserst wichtig ist, wird dadurch unterstützt, dass sowohl PrP^{Sc}, als auch alle toxischen PrP^{C} Mutanten eine ausgeprägte Beeinträchtigung der α-Spaltung aufweisen. Mein Ziel war die Identifikation von PrP^{C} Domänen, die die α-Spaltung ermöglichen, und ihre Charakteristika zu determinieren. Ich habe deswegen eine Vielzahl von PrP^{C} Mutanten kreiert und ihre

proteolytische Aktivität analysiert. Weder die Substitution von Aminosäurenresten an der Schnittstelle, der Austausch oder die Ladungsumkehr von basischen Aminosäuren, Mutation der palindromischen Region oder der Austausch der hydrophobischen Aminosäuren hatten einen grossen Einfluss auf die Spaltung. Auch kleinere Deletionen um die Spaltungstelle resultierten in geringer Beeinträchtigung des Spaltunsprozesses. Das Entfernen grösserer Bereiche hingegen reduzierte die α-Spaltungsrate. Im Falle der Deletionsmutante $PrP^{\Delta(106\text{-}119)}$ tratt praktisch keine Spaltung mehr auf. Daraus schliesse ich, dass die Spaltung von PrP^{C} grösstenteils sequenzunabhängig ist, was mit der proteolytischen Aktivität der γ-Sekretase verglichen werden kann.

Um das zweite Paradigma, PrP^{C} als Modulator der Zellaktivität, beziehungsweise die Fehlregulation seiner Funktion die zum Zelltod führen kann, zu studieren, habe ich die Entwicklung von Lymphozyten untersucht. Ich analysierte die Expression von PrP^{C} während der Lymphocyten-Aktivierung, und der positiven und negativen Selektion von Lymphozyten. Dabei stellte ich eine starke Hochregulation von PrP^{C} in aktivierten T Zellen fest. Ausserdem konnte ich erhöhte PrP-Werte auf aktivierten Lymphozyten, und während der positiven Selektion, und in reaktiven Populationen wie zum Beispiel regulatorischen T Zellen oder B Zellen der Marginalzone nachweisen. Bei der Analyse von *Prnp*$^{-/-}$ und PrP überexprimierenden Mäusen, konnte ich Veränderungen in der Homeostase der Lymphozytenpopulationen feststellen. Ein Grund dafür könnte die Deletion von Zellen mit übermässiger PrP^{C} Expression, während der negativen Selektion von autoreaktiven Zellen, sein. $Prnp^{-/-}$ Lymphozyten hingegen reifen aufgrund von Aktivitätsmangel nicht vollständig. Ausserdem konnte ich zeigen, dass das Co-Stimulationsmolekül CD3 in zirkulierenden T Zellen von PrP^{C} überexprimierenden Mäusen runterreguliert wird, möglicherweise um einen ausgeglichenen Aktivitätszustand der Zelle herstellen zu können, welcher durch die grosse Menge an PrP^{C} reduziert ist. Schliesslich konnte ich zeigen, dass die Überexpression von PrP^{C} partiell die Beeinträchtigung der Entwicklung von CD4 positiven T Zellen verbessern kann. Dafür analysierte ich MHC class II defiziente Mäuse. MHC class II ist wichtig für die Zellaktivierung während der Entwicklung. MHC II defizienten Mäusen fehlen daher CD4 positive Zellen.

Zusammenfassend haben ich hiermit ein Modell entwickelt, das PrP-Funktion und PrP-induzierte Toxizität miteinander vereint, konnte dessen Grundannahmen

bestätigen, und die α-Spaltung eingehend charakterisieren, welche laut unserer Hypothese der Hauptmechanismus ist.

Abbreviations

aa	amino acid
ADAM	a desintegrin and metalloprotease domain
APC	antigen-presenting cell
APP	β-amyloid precursor protein
BCR	B-cell receptor
BM	bone marrow
bp	base-pairs
BSE	bovine spongiform encephalopathy
C1	C-proximal product of α-cleavage
C2	C- proximal product of β-cleavage
CC	charged cluster
CD	cluster differentiation
CD	central domain
CJD	Creutzfeldt-Jakob disease
fCJD	familiar Creutzfeldt-Jakob disease
iCJD	iatrogenic Creutzfeldt-Jakob disease
vCJD	variant Creutzfeldt-Jakob disease
CLP	common lymphoid precursors
CNS	central nervous system
Con A	concanavalin A
CR	complement receptor
CWD	chronic wasting disease
Da	Daltons
DC	dendritic cell
DN	double negative population
DP	double positive population
Dpl	Doppel
EGFP	enhanced green fluorescent protein
ELP	early lymphoid precursors
ER	endoplasmatic reticulum
ETP	early T lineage precursors

FCS	fetal calf serum
FDC	follicular dendritic cell
FFI	fatal familial insomnia
FO	Folicular B-cells
Fr	fraction
GFP	green fluorescent protein
GPI	glycosylphosphatidylinositol
GSS	Gerstmann-Sträussler-Scheinker syndrome
HC	hydrophobic core
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HAS	heat stable antigen
HSC	hematopoietic stem cells
Ig	immunoglobuline
IL	interleukin
ImmatB	Immature B-cells
kDa	kilo Dalton
lck	leukocyte-specific protein tyrosine kinase gene
LCPs	luminescent conjugated polyelectrolytes
Lin	lineage
LN	lymph node
MatB	Mature B-cells
MHC	Major Histocompatibility complex
MZ	marginal zone
N1	N-proximal product of α-cleavage
N2	N-proximal product of β-cleavage
OR	octarepeats
ORF	open reading frame
PBS	phosphate buffered saline
PK	proteinase K
PMA	12-O-Tetradecanoylphorbol-13-acetate
Pre-B	Pre B-cells
Prnd	murine doppel gene
Prnp	murine PrP gene

Pro-B	pro B-cells
PrP	prion protein
PrPase	putative protease complex involved in α-cleavage of PrP^C
PrP^C	cellular isoform of the prion protein
PrP^{Sc}	pathological isoform of the prion protein
SP	single positive
T	transitional B-cells
TCR	T-cell receptor
TdT	terminal deoxynucleotidyl transferase
TME	transmissible mink encaphalopathy
T-reg	regulatory T-cell
TSE	Transmissible spongiform encephalopathy
V-D-J	variable-diversity-joining genes
w.o.	weeks old
Wt	wild-type
wtPrP	unmutated murine PrP^C

1. Introduction

1.1. Prion diseases

1.1.1. Definition of prion diseases

The term “Prion” was first created in 1982 by Prusiner (Prusiner 1982) to characterize a proteinaceous and infectious particle. Therefore, “Prion Diseases” refers to all infectious diseases caused by a nucleic-acid free, self replicating proteinaceous agent. And this is the fundamental postulate of the protein-only hypothesis (Griffith 1967; Prusiner 1982). However, it is still arguable if this infectious agent is completely devoid of nucleic acids and even if this can ever be proved (Adams 1991; Aguzzi and Weissmann 1997; Deleault et al. 2003).

Data available until now strongly suggests that the transmissible agent of all prion diseases in mammals is mainly composed of the toxic isoform of the cellular prion protein (PrP^{C}), named PrP^{Sc}, and its infectivity and toxicity depended on PrP^{C} expression (Büeler et al. 1993; Brandner et al. 1996a). And because these diseases also share the feature of being transmissible spongiform encephalopaties (TSE), the term Prion Diseases has now been popularly associated with transmissible diseases that have PrP^{Sc} as the infectious agent. And this is the definition of “Prion Diseases” I will use in this Dissertation. It is however important to note that there have been growing laboratorial examples of other proteinaceous agents that can form infectious aggregates in mice (Lundmark et al. 2002; Kane et al. 2000; Walker et al. 2002; Xing et al. 2001) and that can even show amyloid strain properties (Xing et al. 2002).

There is also another group of fatal diseases that result from mutations on *Prnp* gene, which encodes for PrP^{C} (Aguzzi et al. 2008a). These diseases are not infectious, and therefore the term “Prion Diseases” is not applicable. For this reason in this Dissertation I will designate them “prionopathies”.

1.1.2. Animal prion diseases

There are several described animal prion diseases. From the natural occurring prion diseases, the origin is still poorly understood, and it is still not clear which diseases had a sporadic origin and which ones resulted from species barrier and further adaptation of the infectious agent.

The earliest recorded descriptions of a prion disease refer to scrapie in sheep and date from the mid 18th century (Aguzzi 2006; Schneider et al. 2008). This name referred to the clinical behaviour shown by sick animals, which scraped or bit areas affected by pruritus (Lampert et al. 1972). The described disease lead to animal death in 3 to 6 months (Cuille and Chelle 1939) and appeared to be acquired by oral-digestive route (Hadlow et al. 1982). However, and unlike other prion diseases, sheep scrapie is considered to be not transmissible to humans (Chatelain et al. 1981; Smith and Bradley 2003).

Other examples of animal prion diseases include Transmissible Mink Encephalopathy (TME) which is a rare prion disease identified in minks in the 1940s (Hadlow 1999) and transmissible to many other mammal species (Marsh et al. 1969; Eckroade et al. 1973). Likewise, Chronic Wasting Disease (CWD) is another fatal prion disease, first identified in the late 1960s in deer (Williams and Young 1980) and later in elk (Williams and Young 1982). Unlike the other most common prion diseases in animals, where the manifestation of the disease is strongly characterized by neurological disabilities, in CWD the clinical signs are more predominantly described as loss of body condition and emanation, and there is a strong component of horizontal transmission through saliva and other secreta or excrements (Sigurdson 2008).

Probably the most dramatic animal TSE is bovine spongiform encephalopathy (BSE). It was first reported in United Kingdom in 1985, and was suggested that it originated from meet and bone food preparations from scrapie infected carcasses or a rare sporadic occurrence of the disease in cattle (Smith and Bradley 2003), although other hypothesis are also considered (Colchester and Colchester 2005). This disease is transmissible to Humans upon contaminated food intake, and is unvariably fatal. The recent world outbreak of an until recently undiscribed disease, also added to the current importance and public awareness about this TSE.

1.1.3. Human prion diseases

The first descriptions of human prion diseases where done in the 1920s by Creutzfeldt and Jakob (Creutzfeldt 1920; Jakob 1921) and therefore named Creutzfeldt-Jakob Disease (CJD). Though very studied because of its particular infectious characteristics, the incidence of this disease is rare, with a global incidence of one to two new occurrences per million people per annum. The most frequent form is the sporadic CJD (sCJD), representing about 75-85% of all human prion cases (Brandel et al. 2000); (http://www.cjd.ed.ac.uk/figures.htm) and generally only manifests itself during adulthood (in the 45-75 year old group), and results in death commonly within 6 months after the onset of the clinical manifestations (Collinge 1997). This disease arises through rare spontaneous misfolding of PrP^{C} into an amyloid form, or through somatic mutations in *Prnp* gene and its incidence is positively correlated with homozygosity at the polymorphic codon 129 of *Prnp*, which encodes either for methionine or valine (Aguzzi et al. 2007a).

Another group of human prion diseases are familial forms which account for around 10% of prion cases in Europe (www.eurocjd.ed.ac.uk). These are heterogeneous diseases acquired through genetic mutations in *Prnp* gene. Although the clinic distinction between these diseases is not clear (Collinge et al. 1992), genetically they are mainly divided into familial CJD (fCJD), which is clinically similar to sCJD, Gerstmann-Sträussler-Scheinker disease (GSS) (Gerstmann et al. 1935), frequently associated with PrP P101L mutation, and Fatal Familial Insomnia (FFI) mainly connected with the D178N substitution (Medori et al. 1992). From these familial cases, fCJD is the most common one, with many attributed hereditary *Prnp* mutations (Gambetti et al. 2003).

Kuru disease (Gajdusek and Zigas 1959; Berndt 1981), means shiver or shake in Fore language, was the first case of a human to human transmission of a prion disease. It was an epidemic circumscribed to the Fore natives in Papua New Guinea, supposedly transmitted through cannibalism (Goldfarb 2002).

The disease that generated highest awareness among the public for the past few years is the variant form of CJD (vCJD). First described in 1996 (Will et al. 1996) it had an earlier onset of disease and a longer clinical course, if compared to other human TSEs. This new disease was later linked to consumption of BSE infected bovine material (Collinge and Rossor 1996; Weissmann and Aguzzi 1997).

A big clinical challenge is related with the various cases of CJD acquired by iatrogenic exposure to human infected material (iCJD), mainly during transplants of cornea (Duffy et al. 1974), dura mater (Thadani et al. 1988), or growth hormone purified from human cadavers (Lazarus 1985). Moreover, the reports about secondary transmissions of prions in blood transfusions (Llewelyn et al. 2004; Peden et al. 2004; Wroe et al. 2006) increased the awareness to the dangerous of the spread of CJD in clinical procedures. These reports stress the need of improvement of diagnostic tools for screening CJD-infected material in donors.

1.1.4. PrP^{Sc} as the prion transmission agent

The first successful proofs of transmission of scrapie were obtained by Cuillé and Chelle (Cuille and Chelle 1939). They were the firsts to perform inoculation experiments in a controlled way and in a time frame long enough to allow them to obtain positive results. The theory that scrapie was caused by a "slow virus" became gradually popular. However, it is important to take in consideration the fact that at the time of those experiments the definition of virus was attributed to an infectious agent that could turn on its own synthesis and replication, which did not imply that a virus should contain nucleic acids. This aspect of the nucleic acid issue was addressed by Alper and colleagues in an experiment that showed that strong UV treatment did not inactivate the infectious agent. The conclusion was that the scrapie infectious agent could not contain more than a 800bp nucleic acid fragment (Alper et al. 1966), speculating that the replicating agent should have a polysaccharide nature (Alper et al. 1967; Field 1966). Following several studies and theories, Prusiner published the Nobel Prize winning "protein only" hypothesis (Prusiner 1982). This theory postulated that the nature of the scrapie infectious agent was proteic. And it went further, by proposing the "heretical" notion that it was uniquely composed of a protein with self replicating properties, denominated "prion", which stood for "proteinaceous infectious". But it was only after the identification of the PrP^{Sc} (Bolton et al. 1982; Prusiner et al. 1983), and the description of the normal PrP^{C} (Oesch et al. 1985; Basler et al. 1986), that the work hypothesis of the mathematician Griffith started to have public relevance (Griffith 1967). Indeed he was the first one to propose a consistent model describing the scrapie agent as a protein. But his model went further than Prusiner's, because all explanations Griffith proposed implied that the prion protein had to be host encoded. He speculated that the scrapie agent is a seed

of modified protein, and that the native host encoded protein attaches to this seed, which eventually grows and divides itself into new seeds. Though this model was published 15 years before the publication of the Nobel Prize awarded protein-only hypothesis (Prusiner 1982), it had stronger assumptions that are now very close to the currently accepted principles. PrP^{C} is protease sensitive, detergent soluble, host encoded and its expression is independent of scrapie infection (Oesch et al. 1985; Basler et al. 1986). In contrast, PrP^{Sc} is the protease resistant core that is specifically associated with prion disease (Meyer et al. 1986).

The next phase of prion research started with experiments focusing on PrP^{C} transgenic mice. Scott and coworkers showed a positive correlation between prion incubation time and transgenic PrP^{C} expression (Scott et al. 1989). Later on, it was discovered that *Prnp*$^{0/0}$ mice, devoid of PrP^{C}, were resistant to scrapie and did not propagate its infectivity (Büeler et al. 1993; Prusiner et al. 1993; Sailer et al. 1994). But the most important evidence for the role of PrP^{C} in prion diseases was the finding that *Prnp*$^{0/0}$ brain tissue was healthy and did not propagate prions, even when in contact with scrapie infected brain graft (Brandner et al. 1996a); and that PrP^{C} was determinant for the spread of infectivity (Brandner et al. 1996b).

The remaining supporting research for the protein only hypothesis arose with the controversial experiment that claimed the conversion of recombinant PrP^{C} fibers into synthetic mammalian prions (Legname et al. 2004; Couzin 2004), and also the amplification *in vitro* of PrP^{Sc} in healthy brain homogenates by protein misfolding cyclic amplification (PMCA) (Castilla et al. 2005; Saa et al. 2006). The latest advances in generating PrP^{Sc} *in vitro* from a synthetic non infectious material were provided with the still arguable results from *de novo* generation of scrapie using PrP^{C} and a mixture of lipids and a synthetic polyanion (Deleault et al. 2007; Aguzzi et al. 2008a). Despite these efforts, it is believed that it is scientifically impossible to disprove that a virus, and not PrP^{Sc}, is the true scrapie infectious agent (Aguzzi and Weissmann 1997) and therefore to prove the protein only hypothesis.

1.1.5. PrP^{C} conversion into PrP^{Sc}

Structural studies on PrP molecule suggest that around 40% of PrP^{C} molecule is organized in an α-helical structure, and 3% in β-sheet conformation (Pan et al. 1993). In contrast, PrP^{Sc} has a β-sheet content of about 40%, and 30% α-helix (Pan et al.

1993; Safar et al. 1993). Given these two distinct structural forms of the same protein, there are intriguing questions to be asked: How does this transition from PrP^{C} to PrP^{Sc} occur? What is the driving force for this sudden β-sheet conformation acquisition? Is it an all or nothing effect of is there a stable intermediate conformation?

There were many studies performed with PrP fibrils, aiming to structurally describe the transition of PrP^{C} into PrP^{Sc} (Stohr et al. 2008), but these studies are far from being clarifying. This is because it is still debatable if these fibrils are infectious (Couzin 2004; Aguzzi et al. 2008a), and also due to the fact that only part of the structure of PrP^{C} is known (Riek et al. 1996; Hornemann et al. 1997; Knaus et al. 2001). There are however two main theoretical models for PrP^{C} conversion into PrP^{Sc} (Figure 1.1) (Weissmann 1999).

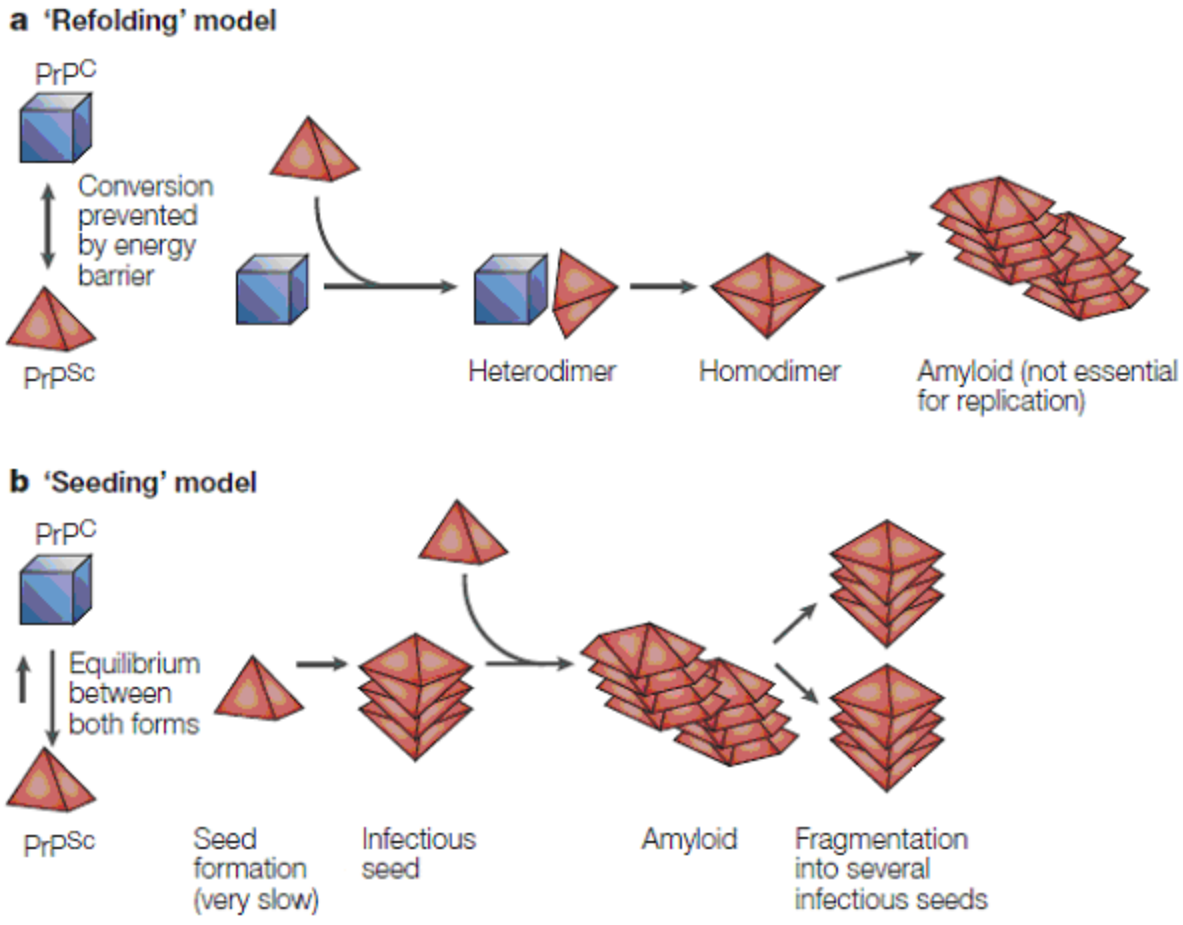


Figure 1.1: Main conceptual models for conversion of PrP^{C} into PrP^{Sc}. A) The Refolding Model; **B)** The Seeding Model. Adapted from (Aguzzi and Sigurdson 2004).

The refolding model proposes that there is a high energy barrier between the PrP^{C} and the PrP^{Sc} conformations and that the former only converts into the later upon interaction with PrP^{Sc}, who serves as a template (Figure 1.1 A). It also requires the help of a chaperone and an energy source, in order to support the partial unfolding of PrP^{C} and refolding into PrP^{Sc} (Prusiner 1991). This process should be repeated in a slow passed way, until large PrP^{Sc} aggregates are formed.

In contrast, the seeding model, proposes that PrP^{C} conformation coexists in equilibrium with the PrP^{Sc} conformation or with one of its precursors. It assumes that this PrP^{Sc} structure is unstable and is stabilized only when aggregated in an oligomeric form that functions as a seed. It also proposes that the formation of this seed is a very rare event, because it demands the presence of a substantial number of molecules, but when it is formed, monomeric PrP^{Sc} units are added on a fast pace and result in the formation of big fibrils that eventually break and generate new seeds. This model is the one that appears to be supported by most of the experimental data (Gajdusek 1988; Brown and Gajdusek 1991; Jarrett and Lansbury 1993), however this question is still far from being resolved (Aguzzi and Sigurdson 2004), and there is the possibility that the biological aggregation of PrP^{C} into PrP^{Sc} works in an intermediate form between these two models.

1.1.6. Prion strains

Like viruses and other pathogens, the property of having different strains is also attributed to prions. This has been suggested already, before the proposition of the protein only hypothesis (Griffith 1967; Prusiner 1982), and resulted from studies where inoculations of different scrapie isolates were done into inbreed animals (Pattison and Millson 1961; Dickinson and Meikle 1971; Bruce and Dickinson 1987). These strains could be characterized by their specific clinical, biochemical and histopathological features in genetically identical hosts. Along with these properties, prion stains have also specific host tropisms, lymphotropic or neurotropic (Aguzzi 2004; Aguzzi et al. 2007b), differential infectivity to other species (Caughey 2003), and most importantly, these individual strain qualities are very stable upon serial passage in inbreed animals (Aguzzi et al. 2007a).

Because in all infectious entities, strain specifications are phenotypes coded by their genomes, it has also been suggested that prion structural properties are

specified by a nucleic portion that is part o the prion (Bruce and Dickinson 1987), by a host gene (Weissmann 1991), or some sort of host structure defining non-nucleic-acid component (Prusiner 1991), but so far there is little evidence supporting these explanations. The alternative and currently more plausible model is the existence of PrP^{Sc} seeds with diverse strain specific conformations, acting as templates for *de novo* folding of PrP^{C} into PrP^{Sc} (Bruce et al. 1991). Though the explanation for the formation of these distinct seeds remains elusive and little is known about the biology of prion strains, advances have been made in their typing. More recently, this typing has been mainly done using biochemical methods that function as a read out for the structure of prions. The most popular classification was based on the PrP^{Sc} fragments and proportion of its abundance upon PK treatment (Collinge et al. 1996; Parchi et al. 1996; Aguzzi et al. 2007a) and it was found that this strain typing has a good match with the clinical histopathological data (Parchi et al. 1999). But more recently a new advance has been made, using luminescent conjugated polyelectrolytes (LCPs). These particles appear to bind differentially to prion strains and to emit a wavelength spectrum that can be a finger-print of a polymeric aggregate (Nilsson et al. 2005; Sigurdson et al. 2007).

1.1.7. Pathological features of prions

The main pathological features of prion diseases are neuronal loss, vacuolation, reactive astrocytosis, and microgliosis (Crozet et al. 2008). In addition, PrP aggregates are frequently present mainly in white matter brain areas such as corpus callosum or the fibre tracts of striatum (Taraboulos et al. 1992a; Bendheim et al. 1992), and these aggregates have generally a high degree of proteinase K (PK) resistance (Prusiner 1982; Aguzzi et al. 2007b). The early effects of prion pathogenesis are dependent on the availability of PrP^{C}, because PrP^{Sc} is not toxic to *Prnp*$^{-/-}$ tissue (Brandner et al. 1996a; Brandner et al. 1996b), and the depletion of host PrP^{C} expression in a scrapie infection context prevents disease and reverses spongiosis (Mallucci et al. 2003) and the early cognitive deficits (Mallucci et al. 2007).

Among the referred early effects of prion pathogenesis, there occurs mainly synaptic loss (Cunningham et al. 2003), dendritic atrophy (Jamieson et al. 2001), and neuronal impairment with loss of GABAergic neurons and alteration of

neurotransmitters (Goudsmit et al. 1981; Diez et al. 2007; Bassant et al. 1986), which consequently result in neurocognitive deficit (Bareggi et al. 2003; Fraser et al. 2003).

1.2. Characterization of PrP^C

1.2.1. Genetic characterization of PrP^C

The PrP^C coding gene is *Prnp*, localized at the short arm of chromosome 20 in humans, and in the homologous region of chromosome 2 in the mouse (Sparkes et al. 1986). This gene is present and conserved in mammals, and in all cases its open reading frame (ORF) is contained within a single exon (Basler et al. 1986; Westaway et al. 1987), which in turn excludes the possibility of existence of alternative RNA splicing events. In hamsters the 5'-noncoding region contains an intron with about 10 kb, and the ORF and 3' untranslated region are in exon 2 (Basler et al. 1986). Mouse and sheep *Prnp* has another exon in the 5' noncoding region, and exon 3 is analogous to the hamster exon 2 (Westaway et al. 1991; Westaway et al. 1994a). About the promoter regions, the specific delimitation of these domains is not known and no TATA-box was found, but upstream the ORF these are GC-rich repeats that are believed to participate in promoting the *Prnp* transcription.

In terms of gene conservation, PrP^C sequence shows a high degree of conservation within mammals (Figure 1.2.) and similar prion proteins were also identified in other vertebrates such as fish (Suzuki et al. 2002; Rivera-Milla et al. 2003), birds (Gabriel et al. 1992), reptiles (Simonic et al. 2000) and amphibians (Strumbo et al. 2001), but never found in invertebrates (Aguzzi et al. 2008a). Though the sequence identity between all these non-mammalian PrP^C molecules is limited, their structure appears to be highly conserved (Aguzzi et al. 2008a). Globally this suggests for an important function of PrP^C, particularly in mammals, where the conservation is higher. However, this does not appear to be in accordance with experimental data (Büeler et al. 1992).

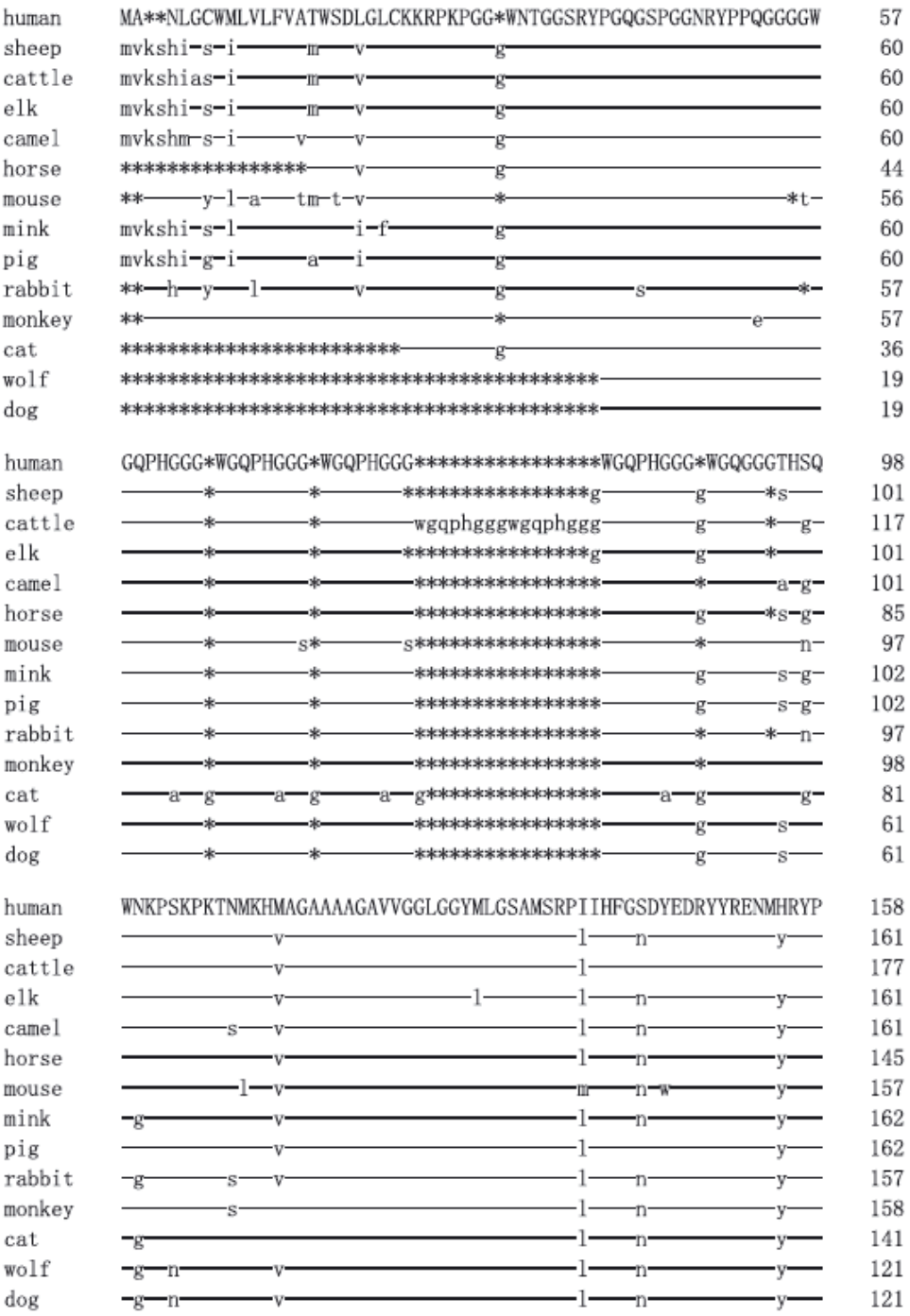


Figure 1.2: Protein sequence alignment of PrP from multiple mammals. Short lines show identical residues and asterisks indicate deletions. The protein residues that differ from Human PrP are indicated explicitly. Adapted from (Wu et al. 2006b).

1.2.2. Characterization of the structure of PrP^C

The PrP^C molecule is a GPI-linked extracellular membrane protein (Stahl et al. 1987) localized in cholesterol-rich lipid rafts of the cellular membrane (Taraboulos et al. 1995; Kaneko et al. 1997a). It is composed by a C-proximal globular domain containing 3 α-helices and 2 short β-sheets, and a N-proximal flexible domain, with an until now unresolved structure (Riek et al. 1996) (Figure 1.3). The N-terminus contains a signal sequence that is cleaved in endoplasmatic reticulum (ER), followed by a short charged domain that is reported to be important for PrP^C internalization (Sunyach et al. 2003). Between amino acids (aa) 51-91 in mouse sequence, there is a series of 5 charged octarepeat sequences (OR), that where reported to interact with Cu^{2+} (Miura et al. 1999; Viles et al. 1999). It follows a small charged cluster (CC) and a large hydrophobic core (HC), which together form the central domain (CD). Between this cluster and hydrophobic core, PrP^C is frequently cleaved, around the residues 110K↓H111 in human sequence or 109K↓H110 in mouse sequence (Chen et al. 1995), designated as the α-cleavage and the α-cleavage site respectively (Mange et al. 2004). This cleavage has been speculated to be a key regulator of PrP function or malfunction (Jimenez-Huete et al. 1998; Checler and Vincent 2002). There is also another important cleavage site, designated as β-cleavage site (Mange et al. 2004), localised several residues upstream (Caughey et al. 1989; Chen et al. 1995). The C-proximal portion of PrP^C is tighten by a disulfide bond and has glycosylation sites (Liao et al. 1986; Kretzschmar et al. 1986; Haraguchi et al. 1989). In the C-terminal part there is a sequence that is cleaved to give rise to a glycosyl phosphatidyl inositol (GPI) anchor (Stahl et al. 1987). Together, unprocessed murine PrP^C is 254 aa long, and is later metabolised into a 209 aa long protein, with 25KDa, which can also be increased by 6 or 12KDa, depending if the molecule is un- mono- or diglycosylated respectively.

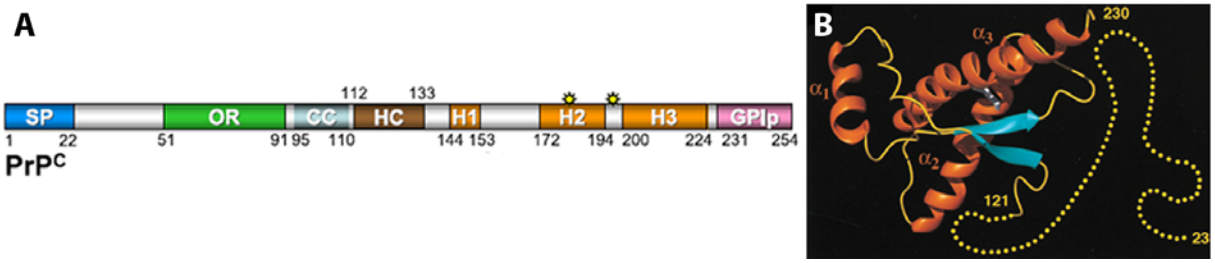


Figure 1.3: Structure of PrP^C molecule. A) Schematic representation of mouse PrP^C sequence, with representation of the main PrP domains: SP, signal peptide; OR, octarepeats; CC, charged cluster; HC, hydrophobic core; H1, helix 1; H2, helix 2; H3, helix 3; GPIp, GPI anchor signal peptide. Yellow stars indicate the glycosylation sites at aa180 and 196. Adapted from (Linden et al. 2008). **B)** NMR structure of human PrP^C. yellow dots represent a region with flexible unresolved structure. Adapted from (Zahn et al. 2000).

1.2.3. General PrP expression profiles

The principal compartment for PrP^C expression is the brain, especially in the grey matter where it is mainly prominent in neurons (Taraboulos et al. 1992a), but it also present in high levels in astrocytes and oligodendrocytes (Büeler et al. 1992).

In the periphery, PrP^C is also widely expressed, with higher evidence in the lymphatic tissues particularly in cells like Follicular Dendritic Cells (FDCs) (Ford et al. 2002; Kranich et al. 2008), heart, skeletal muscle, kidney, lungs and gut (Ford et al. 2002). Moreover, PrP^C expression can be strongly increased by inflammatory conditions (Heikenwalder et al. 2005), suggesting that virtually PrP^C can be highly expressed in any tissue.

1.2.4. Biosynthesis and trafficking of PrP^C

PrP^C synthesis is targeted to the ER, by its N-terminal 22aa long signal sequence, which is later cleaved. After synthesis, the protein travels through the Golgi (Taraboulos et al. 1992b) to the lipid rafts at the cell membrane (Taraboulos et al. 1995; Kaneko et al. 1997a). Before that, the protein is folded and post-tranlatedly modified with the formation of a disulfide bond, the adjunction of a GPI anchor (Stahl et al. 1987) and the possible addition of N-linked oligosaccharide chains (Haraguchi et al. 1989). In the membrane, PrP^C is internalized within few minutes (Sunyach et al. 2003) and in few hours cycles between the cell surface and the endocytic compartment (Caughey et al. 1989; Shyng et al. 1993). The internalization appears to occur via clathrin-coated pits and vesicles (Shyng et al. 1994; Laine et al. 2001), or by caveosomes in non neuronal cells (Peters et al. 2003), or by other mechanisms like bulk flow into endocytic organelles within 16-18h time (Morris et al. 2006). Inside the cell, PrP^C has been found in many organelles (Campana et al. 2005), but most likely it goes to recycling endosomes (Sunyach et al. 2003). During all these

processes, PrP^C can be shedded by α or β cleavage, with a reported rate of about 1% cleavage per hour (Shyng et al. 1993), or by cleavage in the GPI anchor (Borchelt et al. 1990). The sub-cellular localization of α and β cleavage has still not been clarified, although reports suggested that it occurs in an acidified endocytic compartment (Shyng et al. 1993).

1.2.5. The physiological function of PrP^C

The biggest mystery in the prion field is the reason why a protein ubiquitously expressed in the body (Ford et al. 2002), strongly expressed in neurons (Taraboulos et al. 1992a), with homologs proteins within the general vertebrate taxon (Aguzzi et al. 2008a), highly conserved in mammals (Wu et al. 2006a), has a yet non major assigned physiological function. Moreover, it is still very puzzling the discovery that *Prnp*$^{-/-}$ mice do not present any major phenotype (Büeler et al. 1992), apart from being completely resistant to PrP^{Sc} infection (Büeler et al. 1993). However, there have been many minor phenotypes attributed to *Prnp*$^{-/-}$ mice, and many interacting partners assigned to PrP^C molecule (Aguzzi et al. 2008a).

As physiological functions assigned to PrP^C, many studies were performed, suggesting various putative functions for PrP^C. In the nervous system it have been proposed roles of PrP^C in processes such as central nervous system (CNS) development (Steele et al. 2006), spatial outgrowth of neuritis and neuronal survival (Chen et al. 2003; Santuccione et al. 2005), synaptic transmission and reorganization of neuronal circuitry (Colling et al. 1997; Le Pichon et al. 2009), maintenance of Ca^{2+} -activated K^+ currents CA1 pyramidal cells (Colling et al. 1996; Fuhrmann et al. 2006) and modulation of long-term potentiation (Collinge et al. 1994; Manson et al. 1995; Whittington et al. 1995). It has also been implicated both as anti-apoptotic (Zanata et al. 2002; Meslin et al. 2007) and pro-apoptotic agent (Paitel et al. 2003), as well as being reactive to oxidative stress (Brown et al. 1999a) and being able to reduce neuronal excitability and glutamate excitotoxicity (Khosravani et al. 2008). In addition, it has been also suggested a link between PrP^C and resistance to epileptic seizures (Walz et al. 1999) and brain injury (Marciano et al. 2004), brain ischemia (Shyu et al. 2005; Weise et al. 2006), attenuation of neuroinflamation (Tsutsui et al. 2008), neuroprotection (Chiarini et al. 2002), circadian rhythms and patterns (Tobler et al. 1996), memory formation and cognition (Coitinho et al. 2003; Criado et al. 2005),

locomotor activity during exploration of a new environment (Roesler et al. 1999), olfaction (Le Pichon et al. 2009), neuromuscular physiology (Nico et al. 2005) and anxiety generation in stress situations.

PrP^C has also been associated with immune function, namely in supporting self-renewal of hematopoietic stem cells (HSC) (Zhang et al. 2006), early activation in splenocytes (Mazzoni et al. 2005), down-regulation of phagocytosis by macrophages (de Almeida et al. 2005), modulation of T-cell development (Jouvin-Marche et al. 2006), T-cell activation (Mattei et al. 2004) and T-cell immune responsiveness (Bainbridge and Walker 2005; Ballerini et al. 2006).

2. A unifying model for the physiological function of PrP^{C} and toxicity of PrP^{Sc}, Dpl and PrP^{C} deletion mutants

2.1. Introduction

2.1.1. PrP mutations and toxicity

The era of experimental prionopathies started with the generation of PrP^{C} deficient mice. In the cases where the genetic ablation was restricted to the *Prnp* ORF, the knock-out mice were found normal with no altered phenotype (Büeler et al. 1992; Manson et al. 1994), but when large portions of the ORF flanking regions were deleted, mice suffered from Purkinje cell degeneration, which resulted in ataxia (Sakaguchi et al. 1996; Moore et al. 1999; Rossi et al. 2001). This phenotype was later attributed to an accidental over-expression of *Prnd* in the brain, a gene 16Kb downstream of *Prnp* that coded for the PrP-like protein Doppel (Dpl) (Moore et al. 1999; Rossi et al. 2001). Similarly to *Prnp*, *Prnd* is conserved in mammals, with about 25% genetic identity with the globular structured C-proximal half of PrP^{C}, which is reflected by their similar structure (Silverman et al. 2000; Lu et al. 2000; Mo et al. 2001). Dpl is highly expressed in embryogenesis, in brain of newborn mice (Li et al. 2000), in the testis and heart (Moore et al. 1999), suggesting that this toxic protein in adult brain is important for brain development and other physiological processes. Interestingly, mice where PrP^{C} expression was transgenically replaced by Dpl-like PrP^{C} molecules also developed ataxia, cerebellar granule cell loss and a widespread white matter disease (Shmerling et al. 1998; Radovanovic et al. 2005). These molecules were truncated versions of PrP^{C} ($PrP^{\Delta 32-134}$ and $PrP^{\Delta 32-121}$), with deletion of all the N-proximal flexible part, except the N-terminal small charged amino-acids that were claimed to play a role in directing PrP^{C} internalization (Sunyach et al. 2003). Like Dpl, these mutants were composed by an intact globular domain membrane attached by a C-terminal GPI anchor, and with a short positively charged N-terminus, and their toxicity was rescued in a dose dependent manner of PrP^{C} expression. However, transgenic mice bearing shorter deletions in PrP^{C} ($PrP^{\Delta 32-106}$, $PrP^{\Delta 32-93}$ and $PrP^{\Delta 32-80}$) showed no major toxicity in *Prnp*$^{-/-}$ background when transgenically expressed (Fischer et al. 1996; Shmerling et al. 1998). Moreover, PrP^{C} mutants with shorter deletions that encompassed the hydrophobic core region (HC) and the

charged cluster (CC) ($PrP^{\Delta 94-134}$ and $PrP^{\Delta 105-125}$) were strongly pathogenic in the absence of normal PrP^C, and like in the other cases, this toxicity was directly correlated with mutant transgene expression, and inversely correlated with PrP^C coexpression (Baumann et al. 2007; Li et al. 2007). However, shorter deletions in these regions ($PrP^{\Delta 114-121}$ and $PrP^{\Delta 104-114}$) did not appear to be toxic (Baumann et al. 2007; Hegde et al. 1998). More interestingly, transgenic mice with high over-expression of PrP^C also showed neuromuscular toxicity at very old age, although the circumstances were not clearly resolved (Westaway et al. 1994b).

2.1.2. Mechanistic models for neurotoxicity of PrP^C deletion mutants

Many models have been postulated which aimed to explain the mechanisms of toxicity of PrP^C deletion mutants, but the speculative degree of all of them is very high, due to lack of experimental evidences.

One of the first models that aimed to explain the neurotoxicity of PrP^C deletion mutants has come from Shmerling and colleagues. They proposed that there is a ligand for PrP^C that transmits a survival signal when bound to PrP^C. In addition, they also speculated that there is a molecule that mimics the PrP^C domain responsible for interacting with its ligand which then replaces PrP^C in transmitting the survival signal in *Prnp*$^{-/-}$ mice. Finally, Shmerling and coworkers propose that the reason why these PrP^C mutants render toxicity in *Prnp*$^{-/-}$ background is because they out-compete the PrP^C-replacing molecule in binding to its ligand, but are unable to provide the survival signal due to the deletion (Shmerling et al. 1998).

Other models arose with the generation of more deletions in the CC and HC domains (Baumann et al. 2007; Li et al. 2007). One of them, the model from Li (Li et al. 2007), proposed that the binding of PrP^C with its ligand provides a protective signal to the cell, and for this signal it is necessary a region within the CC and HC. When this signal is not present, the result of the binding is toxicity. This damage allegedly depends on the stability of the binding, which is assured by the other PrP^C domains, with especial relevance to the N-proximal ones.

Baumann proposed an alternative explanation, based on studies where not only PrP^C was coexpressed with its deletion mutants, but also when two deletion mutants were coexpressed. In these experiments $PrP^{\Delta 114-121}$ rendered toxicity only when coexpressed with $PrP^{\Delta 94-134}$, but partially rescued the toxicity of $PrP^{\Delta 32-134}$ (Baumann

et al. 2007). This model assumed that PrP^C and its ligand only function in a dimmeric form (Priola et al. 1995). It further proposed that the dimerized ligand is stabilized by its binding to dimerized PrP^C resulting into a survival signal. However, in absence of PrP^C, the ligand is unstable, which results in a mild toxic signal. This toxicity is allegedly further enhanced if the ligand is stabilized by binding to a dominant negative deletion mutant of PrP^C (Baumann et al. 2007).

Another approach to the physiological mechanism for the toxicity of PrP^C deletion mutants was proposed by Hegde and colleagues (Hegde et al. 1998). They proposed that the HC domain of PrP^C can cross the ER membrane (Hegde and Lingappa 1997), and therefore the C-proximal domain or the N-proximal domain can be in the lumen. They also proposed that toxicity is generated in conditions when the C-proximal domain of PrP^C stays in the lumen, which they suggested that happens as a function of the hydrophobicity of the HC region.

2.1.3. Proteolytic processing of PrP^C

The exact localization of α-cleavage site is not yet completely resolved. Studies with PrP^C human brain detected two western blot bands close to each other. These bands were assumed to be the C-proximal product of α-cleavage (C1). Radiosequencing of these bands revealed cleavage sites of 110-111KH↓M112 and 109-110LK↓H111 for each of the two bands (Chen et al. 1995), which corresponds to 110H↓V111 and 109K↓H110 in the murine aa sequence. However, it is questionable that such western blot format would have sufficient resolution to distinguish differences of one amino-acid, which would be 155 Da for a histidine residue. Later, it was shown that 3F4 antibody, which recognises the human 108-111 epitope of PrP (Bolton et al. 1991; Rogers et al. 1991), does not detect any product of α-cleavage, thus suggesting that the cleavage site is within those residues (Jimenez-Huete et al. 1998). Also, *in vitro* experiments incubating recombinant mouse protein with plasminogen detected a C1 product of PrP^C that was identified as starting at aa 110-111HV from mouse sequence (Kornblatt et al. 2003), suggesting that cleavage probably occurred at 109K↓H110. There is another important cleavage, which is more prominent in PrP^{Sc}. It occurs in the OR region (Caughey et al. 1989; Chen et al. 1995; Mange et al. 2004) and is designated β-cleavage (Mange et al. 2004).

The proteases involved in these cleavages are not known and there has been high speculation about this topic. Nevertheless, most studies point to an effect of metalloproteinases in proteolytic processing or PrP^C. For example, Jimenez-Huete (Jimenez-Huete et al. 1998) showed a 75% cleavage inhibition, using 5mM EDTA, and this effect could be rescued by adding Cu^{2+} and Fe^{2+}, indicating that proteolysis of PrP^C could be mediated by Cu^{2+} and Fe^{2+} dependent metalloproteases. Also, *in vitro* studies involving over-expression of this class of enzymes suggested that ADAM10 (ADAM: A Desintegrin And Metalloprotease domain), ADAM17, and probably ADAM9 would play a role in PrP^C cleavage (Vincent et al. 2001; Cisse et al. 2005). However, other studies strongly challenged this data by showing that 10uM of EDTA had absolutely no effect on α-cleavage (Shyng et al. 1993). In contrast, 10uM of the serine protease inhibitor aprotinin substantially reduced C1 generation (Shyng et al. 1993). Also other molecules like plasminogen (Kornblatt et al. 2003) and protein kinase C (Vincent et al. 2000) have been suggested as important factors for PrP^C α-cleavage, and calpain for β-cleavage (Yadavalli et al. 2004). Another study in post-mortem individuals showed a positive correlation between ADAM10 expression and percentage of α-cleavage (Laffont-Proust et al. 2005). However the group only detected the protease in four samples and the specificity of this correlation it was not assessed.

2.1.4. α-cleavage of PrP^C and toxicity of prion diseases and prionopathies

Studies on proteolytic processing of PrP^C and PrP^{Sc} have shown that while the former mainly accumulates C1 cleavage fragment, the latter is cleaved much more upstream, in the β-cleavage site, generating a larger C-proximal product (C2) (Jimenez-Huete et al. 1998). The reason for this difference in cleavage resides in the fact that C1 cleavage site is part of the PK-resistant core of PrP^{Sc} (Caughey et al. 1989; Chen et al. 1995) and that the β-cleavage site is flanking this core (Caughey et al. 1989). However, it is interesting to observe that mice expressing only a deletion mutant form of PrP^C resembling the C1, as $PrP^{\Delta 23\text{-}134}$ and $PrP^{\Delta 23\text{-}121}$, can not replicate PrP^{Sc} (Shmerling et al. 1998; Aguzzi et al. 2008a). An obvious suggestion that arises from these observations is that enhancement of α-cleavage *in vivo* could be a way to

diminish the availability of full-length PrP^C for conversion into PrP^{Sc}. More interestingly is the observation that PrP^C transgenic mutants that have a strong impairment of α-cleavage and C1 generation often show toxicity (Shmerling et al. 1998; Baumann et al. 2007; Fischer et al. 1996; Li et al. 2007). This toxicity is dependent of the transgene expression, and can be rescued in a dose dependent manner by coexpression of normal PrP^C, which is normally cleaved and produces a normal C1 fragment. Together this suggests a correlation between inhibition of α-cleavage and generation of toxicity.

2.2. Description of the model

There have been already many conceptual models that aimed to explain the mechanisms of toxicity of PrP^C mutants (Li et al. 2007; Baumann et al. 2007; Hegde et al. 1998; Shmerling et al. 1998), and many more for toxicity of prions (Aguzzi et al. 2008b; Aguzzi et al. 2008a). But until now, little importance has been given to the hypothetical role of α-cleavage of PrP^C as the main player in PrP^C regulation and toxicity. In this section I suggest a complementary or alternative explanation to the various models that have been proposed until now.

The main focus is the α-cleavage of PrP^C. The model assumes that PrP^C is a modulator of cell activation, as it has been suggested by others (Aguzzi et al. 2008a; Hu et al. 2008; Zomosa-Signoret et al. 2008; Mazzoni et al. 2005). In addition, it is also assumed that the charged amino-acids localized at the N-terminus of the mature PrP^C molecule are important for its localization in a specific membrane environment, which should have an impact on the routes of trafficking of PrP^C and on its function (Sunyach et al. 2003; Shyng et al. 1995; Nunziante et al. 2003; Taylor et al. 2005). Therefore, the cleaved C1 fragment would be devoid of this N-terminus charged domain, and for this reason it should have a different trafficking route, comparing to full length PrP^C (Figure 2.1 A), as it was already shown in the N2a cell culture system (Nunziante et al. 2003).

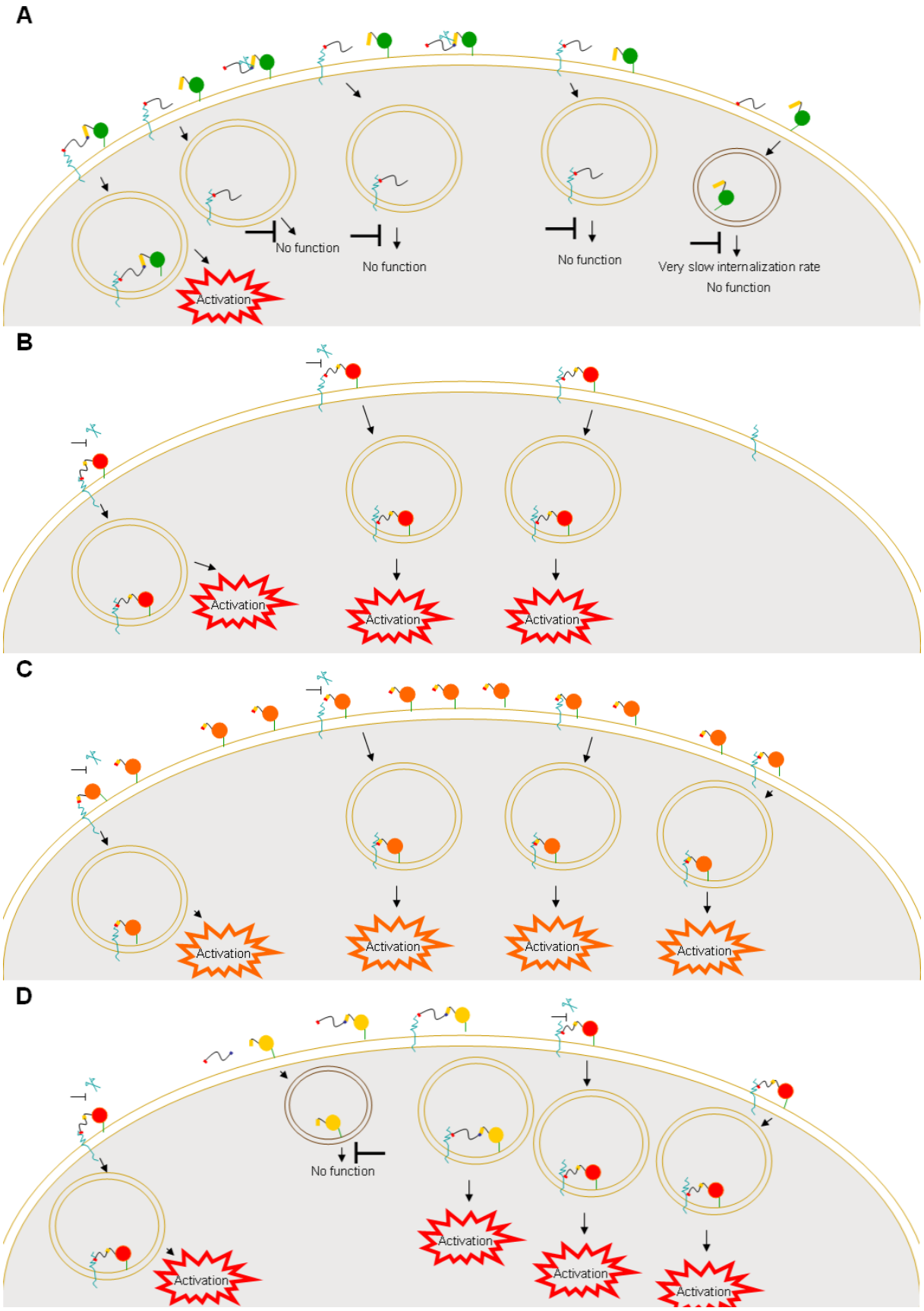
Thus, assuming that the globular domain of PrP^C provides an activation signal when internalized by the coat-pit endocytic pathway targeted by the charged aa in the N-terminus of PrP^C (Sunyach et al. 2003; Shyng et al. 1995; Taylor et al. 2005), the α-cleavage of PrP^C would be a way to separate the supposed C1 signalling domain

from the N-terminus internalization domain. Therefore, cleavage of PrP^C could be an efficient way to inhibit internalization of the globular domain of PrP^C, as suggested by others (Nunziante et al. 2003; Sunyach et al. 2003), and to consequently regulate the cell activation role of PrP^C (Aguzzi et al. 2008a; Hu et al. 2008; Zomosa-Signoret et al. 2008; Mazzoni et al. 2005) (Figure 2.1 A).

2.2.1. Model predictions: the case of prionopathies caused by Dpl and PrP^C deletion mutants

The molecular pattern in PrP^C deletion mutants that *in vivo* rendered the most dramatic toxic effect is a large deletion in the adjacent areas of α-cleavage site, including mainly the CC and the HD domains (Baumann et al. 2007; Li et al. 2007). Mutants bearing these deletions completely abrogated PrP^C cleavage, (Baumann et al. 2007) and resulted in a very strong neonatal toxicity. The proposed model explains these surprising results by predicting that PrP^C mutants, that have an intact effector globular domain and N-terminus charged domain, are constantly internalized through the coated-pit endocytic pathway and thereby provide a constant activatory signal (Figure 2.1 B). The lack of regulation of this activation should result in strong toxicity.

Assuming that PrP^C mutants and the cleaved N1 fragment compete for the same internalization ligand, it is expectable that the toxic effect of the formers can be rescued by coexpression of cleavable PrP^C, in a dose dependent manner. Indeed, the limiting factor of specific ligands of coated-pit mediated endocytic pathways has already been observed with other molecules (Kaplan and Ward 1990; Ward and Kaplan 1990), and if this holds true for PrP^C, it is likely that coexpression of toxic PrP^C mutant and normal PrP^C would result in competition of both molecules for the same ligand. This would finally contravene the toxic effect of the mutant.


A
No function
No function
No function
Very slow internalization rate
No function
Activation
B
Activation
Activation
Activation
C
Activation
Activation
Activation
Activation
D
No function
Activation
Activation
Activation
Activation

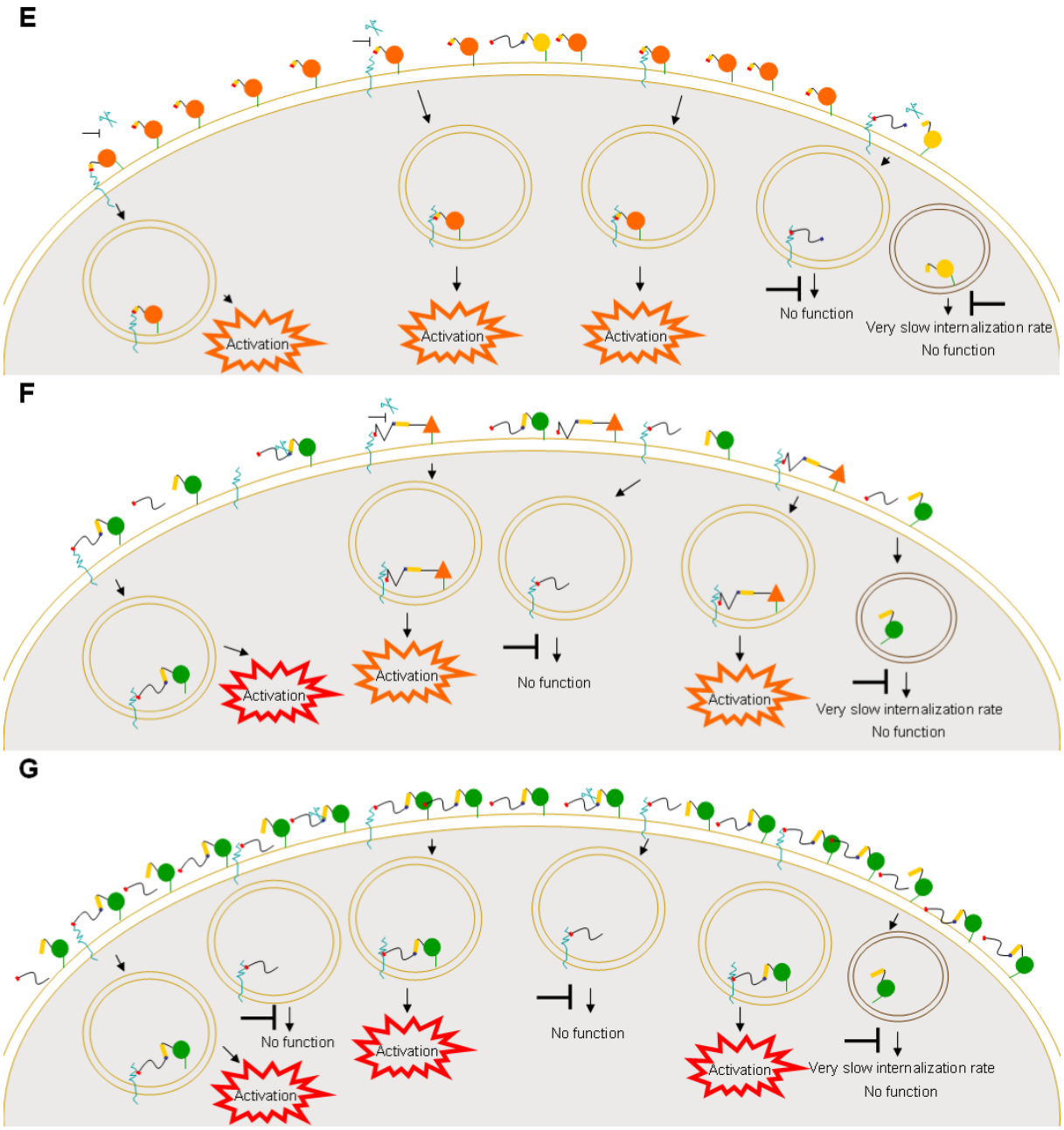


Figure 2.1: Models for PrP-related toxicity. A) Cleavage of PrP^{C} modulates its function: The N-terminus polybasic domain of PrP^{C} interacts with a coated-pit endocytic ligand (Sunyach et al. 2003; Shyng et al. 1995; Nunziante et al. 2003; Taylor et al. 2005), leading to its internalization. When the full length PrP^{C} is endocytosed through the coated-pit dependent pathway, the C-proximal globular domain provides an activation signal. On the other hand, the cleaved N1 competes with PrP^{C} for the internalization ligand and has no effect when endocytosed. Without the N-terminal charges, the C-proximal domain is internalized through an alternative pathway (Nunziante et al. 2003; Sunyach et al. 2003) and provides no activation. This model represents the cellular physiological conditions. **B)** Situation with $PrP^{\Delta 94\text{-}134}$ (Baumann et al. 2007) and $PrP^{\Delta 105\text{-}125}$ (Li et al. 2007) in *Prnp*$^{0/0}$ background. These deletions in the α-cleavage site region inhibit the cleavage, and therefore the full-length form is continuously internalized, providing continuous activation signals, which results in strong toxicity, in spite of their low expression. **C)** Situation with $PrP^{\Delta 32\text{-}134}$ (Shmerling et al. 1998) and Dpl (Moore et al. 1999) in *Prnp*$^{0/0}$ background: These molecules have an N-terminus polybasic domain attached to the globular domain. In these conditions cleavage is abrogated and the total protein is constantly internalized via the polybasic-binding ligand responsible for coated-pit mediated endocytosis. This provides a constant activation signal, which is not very strong, due to binding instability derived from the inexistence of the big portion of the N-proximal domain. (Continues on next page)

D) Situation in scenario of coexpression of the very toxic $PrP^{\Delta 94-134}$ (red) with the mildly toxic $PrP^{114-121}$ (yellow) in *Prnp*$^{0/0}$ background (Baumann et al. 2007). $PrP^{\Delta 94-134}$ is uncleaved and is quickly internalized providing activatory signals. Due to the low expression of this protein, its endocytic ligands are below saturating conditions. The α-cleavage of $PrP^{114-121}$ is impaired and therefore many full-length molecules interact with the free polybasic-binding ligand responsible for coated-pit mediated endocytosis, resulting in a toxic effect additive to the one of $PrP^{\Delta 94-134}$. **E)** Situation of coexpression of the fairly toxic $PrP^{\Delta 32-134}$ (orange) with the mildly toxic $PrP^{114-121}$ (yellow) in *Prnp*$^{0/0}$ background (Baumann et al. 2007). $PrP^{\Delta 32-134}$ is uncleaved (Shmerling et al. 1998) and is internalized by interaction with its N-terminus polybasic-binding ligand responsible for coated-pit mediated endocytosis, providing a constant activation signal. The high expression of $PrP^{\Delta 32-134}$ (Shmerling et al. 1998) saturates the availability of free ligands. When coexpressed with $PrP^{114-121}$, whose cleavage is partially impaired (Baumann et al. 2007), its binding with the N-terminus polybasic binding protein is outcompeted, due to the instability of this interaction, which resulted from the deletion of a big portion of the N-proximal domain. This competition partially rescues the toxicity of $PrP^{\Delta 32-134}$. **F)** Situation of PrP^{Sc} infection (orange triangle). The α-cleavage of PrP^{Sc} is abrogated (Caughey et al. 1989; Chen et al. 1995), resulting in accumulation of uncleaved PrP^{Sc} molecules in the membrane, which compete with cleavable PrP^{C} and N1 for the N-terminus polybasic binding protein, and therefore increases the rate of coated-pit mediated endocytosis of full-length PrP^{C}. This will increase the amount of activatory signals and will result in toxicity. **G)** Situation of strong over-expression of PrP^{C}. When the degree of overexpression of PrP^{C} is extremely high (Westaway et al. 1994b), it can result in the saturation of the α-cleaving proteases, and therefore increase the amount of full-length PrP^{C} coated-pit mediated endocytosis, in comparison with the intake of N1. This can increase the activation status of the cell and finally result in toxicity.

There is an alternative mechanism to the one referred above, which is not exclusive, and may even work together with the afore-mentioned one. This is based on the assumption that the α-cleavage products C1 or N1 transmit an inhibitory signal opposite to the one of full-length PrP^{C}. The result would be that the cleavable form of PrP^{C} would inhibit dose dependently the toxicity of the PrP^{C} uncleavable mutants. This phenomenon of the cleavage product having the opposite function of the full length form of the protein is widely known, especially in the context of the complement proteins (Muller-Eberhard et al. 1969; Manderson et al. 2004), and is a system that provides a good explanation for the absence of phenotype of *Prnp*$^{-/-}$ mice (Büeler et al. 1992).

There are other two important toxic molecules: $PrP^{\Delta 32-134}$ (Shmerling et al. 1998) and Dpl (Moore et al. 1999; Rossi et al. 2001). These are PrP-like proteins, with a GPI-linked globular domain and with the N-proximal domain restricted to an N-terminus polybasic domain (Shmerling et al. 1998; Mo et al. 2001). These show milder toxicity than the ones referred previously, whose deletions are restricted to the CC and the HD domains (Baumann et al. 2007; Li et al. 2007; Shmerling et al. 1998; Moore et al. 1999) (Figure 2.1 C). The reason for this difference in toxicity may possibly result from the fact that molecules with a very short N-proximal tail may have a more unstable binding between N-terminal polybasic domain and its ligand responsible for coated-pit dependent endocytosis.

On the other hand, the observation that expression of the truncation of the globular domain of PrP^C linked to the GPI anchor ($PrP^{\Delta 141-131}$) results in cell toxicity when coexpressed with PrP^C (C. Bridel and A. Aguzzi, unpublished data), can be explained by an aggregation of the mutant molecule with PrP^C, which is expectable given the high amyloid propensity of the HC of PrP^C (Kourie 2001). Such an aggregation could inhibit the α-cleavage and therefore diminish the generation of N1 product. This would lead to a toxic phenotype, dose dependently of PrP^C expression.

2.2.2. Model predictions: a more parsimonic explanation for Baumann's effect

This model can also provide a more parsimonic clarification of Baumann's effect (Baumann et al. 2007) than the one provided in that publication. Baumann based his model on a mutant-ligand affinity hierarchy and on dimmeric property of those molecules, in order to explain the finding that the expression of a $PrP^{\Delta 114-121}$ was innocuous in the presence or absence of full-length PrP^C, but enhanced the toxicity of $PrP^{\Delta 94-134}$, which had low expression (Baumann et al. 2007), and diminished that of $PrP^{\Delta 32-134}$, which was highly expressed (Shmerling et al. 1998).

According to the model I propose here, the simple assumption that in the paradigm of the low expressed $PrP^{\Delta 94-134}$ there is no saturation of the ligand for the coated-pit mediated endocytic pathway, allows the deduction that coexpression of another deletion mutant with partial inhibition of α-cleavage (Baumann et al. 2007), which is not sufficient to generate a toxic phenotype *per se*, would add to the toxicity of $PrP^{\Delta 94-134}$ (Figure 2.1. D). On the other hand, assuming that the high expression of $PrP^{\Delta 32-134}$ saturates all the internalization ligands, it is expectable that coexpression with this PrP^C deletion form, along with the low toxic mutant $PrP^{\Delta 114-121}$ and with some of its N1 cleavage products, results in a competition for the ligand, and therefore would partially impair the toxicity of $PrP^{\Delta 32-134}$ (Figure 2.1 E).

This line of thought can equally be applied to explain the phenotype of secreted PrP^C, $PrP^{\Delta 232-254}$, which is a mutant that lacks the GPI anchor (Chesebro et al. 2005). According to unpublished data from F. Baumann, B. Chesebro and A. Aguzzi, this construct has no influence in toxicity of $PrP^{\Delta 94-134}$ in a *Prnp*$^{-/-}$ background, but partially rescues toxicity of $PrP^{\Delta 32-134}$ in absence of normal PrP^C expression. F. Baumann and

A. Aguzzi showed that $PrP^{\Delta 94\text{-}134,232\text{-}254}$, which is an anchorless form of the highly toxic form $PrP^{\Delta 94\text{-}134}$, shows no phenotype when transgenically expressed in *Prnp*$^{-/-}$ mice (F. Baumann and A. Aguzzi, unpublished results), strongly suggesting that PrP^{C} needs to be membrane attached in order to be functional. This therefore implies that the anchorless $PrP^{\Delta 232\text{-}254}$ should not be functional if internalized. Thus, $PrP^{\Delta 232\text{-}254}$ may probably compete with $PrP^{\Delta 32\text{-}134}$ for the limiting protein responsible for induction of coated-pit dependent endocytosis, which binds the PrP N-terminus polybasic sequence. In addition, $PrP^{\Delta 232\text{-}254}$ may be cleaved outside the cell (Walmsley et al. 2008), and though a cleavage outside the cell membrane should not be very effective in terms of correct localization of its products, these generated N1 fragments could be the competitors with $PrP^{\Delta 32\text{-}134}$ for the internalization ligand. But independently if it is the full length secreted $PrP^{\Delta 232\text{-}254}$, or if it is his N1 product that competes with toxic $PrP^{\Delta 32\text{-}134}$ for the internalization ligand, the end result is that a non-functional molecule competes with a pathogenic one, and this process should partially lead to a rescue of the toxicity of $PrP^{\Delta 32\text{-}134}$.

On the other hand, given the low abundance of $PrP^{\Delta 94\text{-}134}$ in transgenic mice (Baumann et al. 2007), the coexpression of secreted $PrP^{\Delta 232\text{-}254}$ would not be sufficient to saturate the ligands for the PrP N-terminus polybasic region. In this scenario, the function of $PrP^{\Delta 232\text{-}254}$ or of its N1 product would add to the high toxicity of $PrP^{\Delta 94\text{-}134}$. But given the non-functionality of the former molecules, coexpression of these two constructs in transgenic mice would show no difference in toxicity, when compared to $PrP^{\Delta 94\text{-}134}$ expressed alone.

2.2.3. Model predictions: molecular mechanism of toxicity of PrP^{Sc}

Assuming that PrP^{Sc} has a low cleavage rate, with no α-cleavage (Jimenez-Huete et al. 1998; Caughey et al. 1989; Chen et al. 1995), it is predictable by this model that an accumulation of uncleavable PrP^{Sc} in the cell membrane, or a PrP^{C} cleavage inhibition by interacting with PrP^{Sc}, would considerably increase the internalization of the full length form of PrP, and diminish the intake of N1 cleavage product (Figure 2.1 F). The end result is that PrP^{Sc} should induce systemic stress and toxicity in a very slow pace, and that it would only be toxic to cells expressing membrane bound PrP^{C}, which corroborates with existing published data (Büeler et al. 1993; Brandner et al. 1996a; Aguzzi et al. 2007a).

2.2.4. Model predictions: overexpression of normal PrP^C

In conditions where overexpression of PrP^C would be so high that would saturate the availability of PrP^C α-cleavage protease complex, there would be an accumulation of unprocessed PrP^C. According to the proposed model, such accumulation would saturate the coated-pits endocytic ligand for the N-terminus of PrP^C. As consequence, there would be a strong diminishment on regulation of the activatory signalling of PrP^C, which could result in cell toxicity (Figure 2.1 G). Indeed is has already been reported such an effect in brain and in other organs, in mice over-expressing PrP^C (Westaway et al. 1994b; Jouvin-Marche et al. 2006).

Furthermore, an increase of cell activation in PrP^C overexpressing conditions also explains the results suggesting that there is a higher incidence of tumours in mice overexpressing PrP^C (P. Schwarz and A. Aguzzi, unpublished results), for the reason that it has been widely known that forced cell activation increases the probability of tumourgenesis (Tlsty and Coussens 2006; Tibes et al. 2005). Therefore, if overexpression of PrP^C indeed induces activation, which can lead to toxicity, it is also expected that it will increase the probability of generating tumours in aged mice.

2.3. Discussion

As described previously, there have already been proposed models describing the molecular mechanisms of toxicity of modified PrP molecules (Shmerling et al. 1998; Hegde et al. 1998; Baumann et al. 2007; Li et al. 2007; Stefani and Dobson 2003; Brown 2002). However, the vast majority of them only attempt to elucidate either the toxic mechanisms of prionopathies in mice, or of PrP^{Sc}. Moreover, from the ones that address the noxious effect of PrP^C deletion mutants and Dpl, only Baumann incorporated the complex network of toxic effects when $PrP^{\Delta 114\text{-}121}$ is coexpressed with $PrP^{\Delta 32\text{-}134}$ or with $PrP^{\Delta 94\text{-}134}$ (Baumann et al. 2007). Together, this indicates that despite the high likelihood that the same principles govern the effects of PrP^C, PrP^{Sc} and all PrP-like proteins, there have been very few attempts to incorporate toxicity of PrP^{Sc} and prionopathies in one single mechanistic model. It may probably be possible to incorporate all the toxicity of the prion field into an adapted version of

Baumann's framework (Baumann et al. 2007), however this would most likely increase the amount of assumptions and the rigidity of the model, which would be a clear challenge to the law of parsimony.

The proposed model therefore aimed to unify all the toxicity of PrP^{Sc}, PrP^{C} mutants and its homolog Dpl, into one single mechanistically framework. And this may be the most simplistic and flexible set of explanations from all the prion-unifying models. The assumptions supporting it are:

i) The PrP^{C} N-terminus polybasic region binds to a ligand that targets a specific signal, which most likely is a coated-pit mediated internalization pathway. This suggestion is supported by findings from different labs (Sunyach et al. 2003; Shyng et al. 1995; Taylor et al. 2005), and one of the strongest evidences resulted from showing that incorporating this domain in GPI-linked Thy-1 was sufficient to induce a coated-pit dependent endocytosis (Sunyach et al. 2003), like the one shown for PrP^{C}, and deletion of this domain or from PrP^{C} would inhibit this endocytosis (Shyng et al. 1995).

ii) The carboxy-product of cleavage of the N-terminus positively charged amino acids is unable to be internalized by the same pathway as normal full length PrP^{C}, and enters the cell in a very slow manner. Experimentally this has been successfully achieved in the N2a cell model (Shyng et al. 1995; Nunziante et al. 2003), which is a neuroblastoma cell line.

iii) Internalization of the C-proximal globular domain of PrP or of Dpl, via the coated-pit endocytic pathway, generates a cell activation signal. This is the strongest assumption and there is not enough data to support it. On one hand there has been a strong body of evidence suggesting that PrP^{C} participates in cell activation (Aguzzi et al. 2008a; Hu et al. 2008; Zomosa-Signoret et al. 2008; Mazzoni et al. 2005), however, given the innumerous functions and interacting partners attributed to PrP^{C} (Aguzzi et al. 2008a; Zomosa-Signoret et al. 2008), this data lacks further support. Moreover, there has been little data restricting the cellular activation with the globular domain of PrP, maybe because it would demand to use a construct with only the C1 and the N-terminus polybasic domain to target it to the right internalization pathway. The best examples of such molecules are $PrP^{\Delta 32\text{-}134}$ and Dpl. While there have been few cell activation studies done with the former protein, the finding that Dpl is highly expressed in development tissues, including brain of newborn mice (Li et al. 2000),

may indicate for activatory role, and thence that the globular domain participates in cell activation. However this statement lacks further verification.

iv) Another fundamental assumption is that the amount of the ligand for the PrP N-terminus polybasic domain is limited. Because such a ligand has not been identified so far, it is difficult to support this assumption. However, it is already established that such endocytic pathways are strictly regulated (Mellman 1996; Kaplan and Ward 1990; Ward and Kaplan 1990). This assumption goes further, by assuming that expression of the most toxic constructs is bellow saturation of this ligand, and that the availability of $PrP^{\Delta 32\text{-}134}$ is so high that saturates the binding for the ligand of the N-terminal positively charged domain. Although lacking experimental data, these assumptions are very reasonable, given the very low and the very high expression of the most toxic and the less toxic constructs, respectively (Shmerling et al. 1998; Li et al. 2007; Baumann et al. 2007).

v) The regulation of the amount of α-cleaving protease available to cleave PrP^{C} is another assumption. This is expectable given the fact that proteolytic pathways are usually strictly regulated (Ehrmann and Clausen 2004). The consequent result is that in high PrP^{C} overexpressing conditions, it is possible that occasionally the amount of this protein would saturate the available proteases.

vi) The assumption that molecules with a long N-proximal tail are more stable in terms of providing activatory signals or binding with the endocytic ligand, also remains to be proved. However, the most parsimonic explanation for the difference in toxicity between the highly toxic PrP^{C} mutants with deletions restricted to the CC and HC (Baumann et al. 2007; Li et al. 2007), and the lower toxicity of constructs with almost all N-proximal domain deleted (Shmerling et al. 1998), may be the higher stability of the formers.

vii) In order to incorporate unpublished results obtained from experiments with mutants expressing only the N-proximal PrP^{C} domain linked with a C-terminus GPI ($PrP^{\Delta 141\text{-}131}$; C. Bridel and A. Aguzzi, unpublished data), It is proposed that such a flexible and highly amyloidogenic molecule (Kourie 2001) targeted to the same membrane microdomains of PrP^{C} would generate an association between the N-proximal part of these two molecules, and consequently impair accessibility to the PrP^{C} α-cleavage site.

viii) The other postulation necessary to incorporate unpublished data from F. Baumann, B. Chesebro and A. Aguzzi, is that a PrP^{C} molecule needs to be

membrane bound by the GPI anchor, in order to exert its function. F. Baumann and A. Aguzzi showed already that $PrP^{\Delta 94\text{-}134,232\text{-}254}$, which is the anchorless version of the highly toxic $PrP^{\Delta 94\text{-}134}$, has no toxicity, and this supports the proposed hypothesis.
ix) The final consideration is that the anchorless versions of PrP^{C} (Chesebro et al. 2005) and the N1 product of its α-cleavage (Walmsley et al. 2008) can be in such a concentration and proximity to the membrane, that allows them to be internalized by binding the ligand for the PrP^{C} N-terminus polybasic residues. The parallel of this mechanism with the specific binding of several extracellular proteins to its cellular receptor suggests that the secretion of PrP^{C} does not inhibit the ligation of this molecule to certain interacting partners at the membrane.

Given all these assumptions, particularly the first three, the natural conclusion is that α-cleavage of PrP^{C} is a crucial way to regulate the function of this protein, and as a result, to modulate its own potential toxicity.

2.4. Outlook

As discussed above, from all the assumptions of this model, the most critical one is the cellular activatory property of PrP^{C} (assumption “iii”). Although extensively studied (Aguzzi et al. 2008a; Hu et al. 2008; Zomosa-Signoret et al. 2008; Mazzoni et al. 2005), this question demands further analysis, and new scientific approaches. This issue is addressed in more detail in section 4 of this dissertation.

The main regulating mechanism of this model is the cleavability of the PrP-related proteins, and the toxicity that results from cleavage impairment. Therefore this question deserves further detail. For that purpose section 3 aims to discuss the physiological relevance of α-cleavage of PrP^{C}, and suggests further studies that will add to the present knowledge in the field. Moreover, a careful study to elucidate the sequence domains in PrP^{C} sequence and the main biochemical factors that regulate α-cleavage has been attempted.

Additionally, there is also the plan to further validate experimentally these assumptions, and the model itself. However, the most important step to verify this model is to identify the main variables and the mathematical relations that link them, so that it could be possible to numerically predict the degree of toxicity of further constructs. This is an on going study.

3. Candidate approach study for the main domains in PrP sequence that regulate its cleavage at 109KH110

3.1. Introduction

3.1.1. Aim of the study

As illustrated in the model described ahead in this document (Figure 2.1), all the mechanisms of toxicity of PrP and PrP-related proteins can be explained by an impairment of α-cleavage and consequent lack of regulation of PrP dependent cell activation signalling. The main support for this idea comes from work that has been performed on the coated-pit dependent endocytic pathway that internalizes PrP^C by interaction with its N-terminal polybasic region (Sunyach et al. 2003; Shyng et al. 1995; Taylor et al. 2005). Hence, given the probably crucial role of α-cleavage in regulating the toxicity of PrP-derived molecules, an analysis of α-cleavage was performed. The main focus was to assess the domains in PrP^C sequence which are important for its cleavage. Because the α-cleavage occurs in the flexible "unfolded" region of PrP^C, it is reasonable to rationalize that the recognition domain for this proteolysis should be a continuous region in the vicinity of the cleavage site. Therefore, the identification of such continuous unfolded recognition region, together with the already available knowledge of the localization of the α-cleavage site (Chen et al. 1995; Kornblatt et al. 2003), may be a crucial tool for biochemically design molecules that should enhance or block α-cleavage. Because the cleavage products of α-cleavage do not appear to be convertible to PrP^{Sc} (Shmerling et al. 1998; Aguzzi et al. 2008a; Caughey et al. 1989), the identification of a substance that could specifically cleave PrP^C would be of important clinical value as anti-PrP^{Sc} therapy. In addition, the identification of the binding domain in PrP^C for its α-cleavage protease complex, here called PrPase, would allow to structurally screen for specific interacting partners and for consequently identify the molecules responsible for this physiological cleavage, and hopefully to gain insights on the pathways involved in PrP^C function.

For this purpose several PrP^C constructs were designed, with several mutations predominantly in the PrP^C CC and HC domains, which are the regions that flank the α-cleavage site. Each one of these new molecules was used to address a question

about the principal domains in PrP^C and its main biochemical features, which are responsible for modulating the physiological proteolysis of PrP^C. The degree of cleavage of these constructs was assessed mainly in Hpl cells, a cell line purified from the hippocampus of a *Prnp*$^{-/-}$ mouse (Kuwahara et al. 1999).

3.1.2. Previous studies performed on α-cleavage of PrP^C mutants

Though the need to assess the main domains in PrP^C molecule that are responsible for modulating its cleavage may be one of the priority questions in the prion field, studies assessing α-cleavage of PrP^C mutants are very scarce. From the experiments performed in mouse model, it is noteworthy that all constructs that were not cleaved were shown to be toxic when transgenically expressed in mice (Shmerling et al. 1998; Baumann et al. 2007; Li et al. 2007).

Although not highlighted by the authors, Shmerling and coworkers were the first to show a correlation between absence of α-cleavage, and neurotoxicity of PrP^C constructs in *Prnp*$^{0/0}$ background (Shmerling et al. 1998). In this pioneer study of α-cleavage of PrP^C mutants in mice, many transgenic lines were created expressing PrP^C deletion mutants conserving the C-proximal globular domain, the N-terminus polybasic region, and varied portion of the N-proximal domain. The authors showed that $PrP^{\Delta 32\text{-}134}$ and $PrP^{\Delta 32\text{-}121}$ were toxic in *Prnp*$^{0/0}$ background, and were not cleaved. Another construct, $PrP^{\Delta 32\text{-}106}$ had a partial impairment in its cleavage, and did not cause toxicity during the experimental time. Finally, $PrP^{\Delta 32\text{-}93}$ and other shorter deletions within these amino acids were normally processed and showed no toxicity.

Apart from that report, only two other studies accessed cleavage of constructs with deletions around the α-cleavage site. $PrP^{\Delta 94\text{-}134}$ and $PrP^{\Delta 105\text{-}125}$ had a complete cleavage inhibition and were highly toxic in *Prnp*$^{0/0}$ background (Baumann et al. 2007; Li et al. 2007), and $PrP^{\Delta 114\text{-}121}$ was proteolyticaly processed with low efficiency and showed very mild toxicity (Baumann et al. 2007).

Concerning cell culture systems, the data available is not more abundant. There is one study assessing α-cleavage of PrP^C in constructs with mutations in the palindromic region in the N2a cell culture system (Wegner et al. 2002). It was concluded that $PrP^{GG113,118AA}$ was normally cleaved, but $PrP^{A112,114\text{-}117,119G}$ and $PrP^{\Delta 105\text{-}125}$ were not or very weakly cleaved, a finding that according to the authors, was “highly significant and reproducible” (Wegner et al. 2002).

In human neuroblastoma SH-SY5Y cells, it was assessed the cleavage of murine PrP^{C} constructs with deleted octarepeat region $PrP^{\Delta 51-90}$ or with insertion of more repeats in that region (Watt et al. 2005). There was no apparent difference in C1 generation comparing with unmutated PrP^{C}. These results were reproduced in Hpl cells (Sakudo et al. 2005) using a similar murine $PrP^{\Delta 53-94}$, which also has the octarepeats removed. In this same study, the authors also showed that $PrP^{\Delta 95-132}$ was not cleaved in Hpl cells (Sakudo et al. 2005), agreeing with the uncleavability of $PrP^{\Delta 94-134}$ in mouse brain (Baumann et al. 2007).

The last relevant study assessing α-cleavage of PrP^{C} mutants was performed in a cell culture system expressing ovine PrP (ovPrP) constructs with GFP insertion in the N-proximal region (Tveit et al. 2005). In this system, $ovPrP^{K113R}$, $ovPrP^{K113D}$, $ovPrP^{K113A}$ and $ovPrP^{KHV113-115AAA}$, all fused with GFP, were cleaved in a similar fashion as unmutated $ovPrP^{C}$ with GFP insertion.

These are the few studies that assessed the domains in PrP^{C} molecule that are important for α-cleavage. In spite of the high *in vivo* correlation between toxicity and cleavage impairment, the work performed in this area is very limited, and always on a small scale, which adds importance to this study.

3.2. Results

3.2.1. Characterization of α-cleavage of PrP^{C}

To characterize the processing of PrP^{C}, glycosylation and α-cleavage were assessed in various murine cell lines transfected with a construct coding for PrP^{C} and for the mutant $PrP^{\Delta(32-134)}$, which is reported to have an electrophoretic mobility similar to C1 (Shmerling et al. 1998) (Figure 3.1 A-B). The cell lines used were N2a-PK1 cells, a murine neuroblastoma cell line (Klohn et al. 2003); NIH3T3, a murine fibroblastic cell line (Todaro and Green 1963); Npl cells, a neuronal cell line derived from *Prnp*$^{0/0}$ mice, which do not express PrP (Nishimura et al. 2007); Hpl cells, a cell line derived from hippocampus of *Prnp*$^{-/-}$ mice, which do not express PrP, but express the Doppel protein linked to the *Prnp* promoter (Kuwahara et al. 1999). In all these cell lines PrP^{C} and $PrP^{\Delta(32-134)}$ appeared to be normally glycosylated (Figure 3.1 A). Also, upon removal of the glycosyl groups with PNGase treatment, it was detectable in all cell lines a strong band with similar electrophoretic mobility as $PrP^{\Delta(32-134)}$, and

therefore corresponding to the C1 fragment of PrP^C (Figure 3.1 B). To assess the efficiency of PrP^C cleavage in other cell models, PrP^C and $PrP^{\Delta(111-120)}$ were transfected into human HeLa cells (Gey et al. 1952) (Figure 3.1 C), and into murine primary embryonic fibroblasts (MEFs) derived from *Prnp*$^{0/0}$ mice (Figure 3.1D). This mutant $PrP^{\Delta(111-120)}$ is a molecule that lacks the PrP^C palindromic region which flanks the α-cleavage site, and that for this reason was expected to have an impairment in C1 generation. In both HeLa and MEFs the murine PrP^C molecule was efficiently cleaved (Figure 3.1 C-D). In addition, α-cleavage of the mutant $PrP^{\Delta(111-120)}$ was impaired in both cell systems. Together, these observations indicate that α-cleavage of murine PrP^C is a process common to many cell systems which include murine- and human-derived cell lines and murine primary cells.

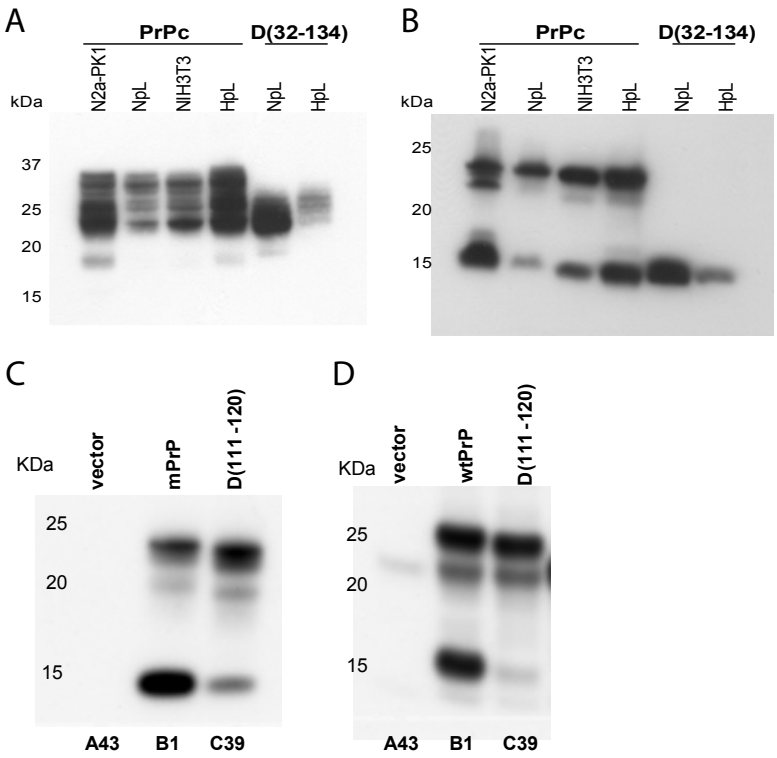


Figure 3.1: Proteolysis of PrP^C in various biological models. A) and **B)** Western blot of cell lysates of various cell lines (N2a-PK1, Npl, NIH3T3, Hpl) untreated **A)** or treated **B)** with PNGase. Cells were transfected with a plasmid coding for PrP^C (lane 1-4) or $PrP^{\Delta(32-134)}$ that lacks the most part of the N-proximal domain (lane 5-6). Detection was done with POM1, which binds the C-proximal region of PrP. **C)** Western blot of PNGase treated cell lysates of human HeLa cells transfected with murine PrP^C molecule and with murine $PrP^{\Delta(111-120)}$. Detection was done with POM19, which is an anti-PrP C-proximal domain antibody that does not react whit human PrP. **D)** Western blot of PNGase treated cell lysates of mouse primary embryonic fibroblasts from *Prnp*$^{-/-}$ mice. Cells were transfected with murine PrP^C molecule and with murine $PrP^{\Delta(111-120)}$. Uncleaved PrP is the band around 25KDa; C1 is the band around 15KDa.

3.2.2. Evaluation of the role of the charged residues neighbouring the α-cleavage site, in the modulation cleavage of PrP^C

Because changes on the proteic secondary structure pattern may correspond to sites for protein binding, cleavage, or for other biochemical alteration, we decided to evaluate the role on α-cleavage of the PrP^C domains localized in the region of the α site. This PrP^C α-cleavage site is located between CC and HC. It is flanked by positively charged residues that extend upstream and has palindrome in the downstream neighbouring region.

I started by evaluating if the positive charges at the α-cleavage site would be a determinant for defining the proteolytic site. For this purpose a series of PrP^C mutants were designed with substitutions of these basic amino-acids. $PrP^{KH109-110AA}$ had the two charged amino acids mutated to small neutral alanine residues, and $PrP^{KH109-110DD}$ had the charges inverted to negative ones, by mutation to aspartic acid (Figure 3.2 A). As control, the mutant $PrP^{\Delta(32-134)}$, that has a C1-resembling electrophoretic mobility (Shmerling et al. 1998; Baumann et al. 2007), was used. The cleavability of these constructs was determined by assessing the C1 generation on Hpl and Npl cells (Figure 3.2 B). For this, cell lysates of these cell lines transfected with PrP^C mutants and PrP^C were collected. PrP^C glycosylation and proteolytic processing were then assessed in PNGase untreated and treated samples respectively. In both cell lines, $PrP^{KH109-110AA}$ and $PrP^{KH109-110DD}$ and the large deletion mutant $PrP^{\Delta(32-134)}$ were glycosylated in a similar fashion as PrP^C. This data suggests that charge inversion or large deletions in the flexible N-proximal domain of PrP^C do not have a strong impact in PrP^C glycosylation (Figure 3.2 B), as in accordance with other studies (Baumann et al. 2007; Shmerling et al. 1998; Li et al. 2007). In addition, in both Hpl and Npl cells the degree of α-cleavage of $PrP^{KH109-110AA}$ and $PrP^{KH109-110DD}$ was comparable to unmutated PrP^C (Figure 3.2 B). This indicates that abolishment and even inversion of the positive charges at the α-cleavage site do not appear to inhibit α-cleavage. Furthermore, it suggests that the pattern of proteolytic processing of PrP^C that is seen in Hpl cells is reproducible in other cell types, as Npl cells. For this reason, this study focused mainly in only one cell system, which was the Hpl cells derived from hippocampus of *Prnp*$^{-/-}$ mice.

To further explore the observation that suggests that α-cleavage is independent of the charges and sequence of the cleavage site, I designed a construct that had removed the five charges in the region of the α-cleavage site. For this, the mutant $PrP^{K100,103,105,109A\ H110A}$ was constructed, which had five positive histidine and lysine residues mutated to uncharged alanines (Figure 3.2 A). In addition I evaluated if swapping the position of the α-cleavage site would alter the proteolytic processing of PrP^{C}. For this, the mutant $PrP^{KHVA109\text{-}112VAKH}$ was elaborated, which had the α-cleavage site shifted downstream by two amino-acids (Figure 3.2 A). As with the previously assessed constructs, $PrP^{K100,103,105,109A\ \ H110A}$ and $PrP^{KHVA109\text{-}112VAKH}$ generated C1 in a frequency comparable to the one shown by unmutated PrP^{C}.

The next step was to assess the degree of α-cleavage upon stronger alterations on the CC. For this purpose two constructs were designed. $PrP^{H95,110E;\ K100,103,105,109E}$ had all positive six residues from the CC mutated to negative glutamic acid, resulting in a complete inversion of all the charges (Figure 3.2 A). $PrP^{K100,103,105,109E}$ had the four positive lysines from the CC reversed to glutamic acid residues, and therefore these mutated acidic residues were mixed with two basic histidines (Figure 3.2 A). Simultaneously it was tested again the construct $PrP^{K100,103,105,109A;\ H110A}$, which had removed the charges in the region of the α-cleavage site, and replaced to uncharged alanines (Figure 3.2 A). Comparison of the proportion of C1 generated in these constructs, with the C1 generated in unmutated PrP^{C} suggested that, as discussed before, removal of the five positive charges from the α-cleavage site and in the neighbouring region had no remarkable effect on the proteolytic processing of PrP^{C} (Figure 3.2 D,F). In addition, a strong modification as the inversion into negative, of four or even the six positive charges of the α-cleavage site region, resulted in only about 38% impairment on proteolysis of PrP^{C} (Figure 3.2 D,F). These results suggest that the positive charges of the α-cleavage site and from its neighbouring regions play no important role in modulating proteolysis of PrP^{C}. However, the overall polarity of the CC appears to have a partial impact in α-cleavage, because inversion of the charges resulted in about 38% impairment of PrP^{C} cleavage. Furthermore, this data shows that α-cleavage of PrP^{C} is strongly sequence independent, because amino-acid shift, charge removal, and charge inversion of the amino-acids reported to flank the α-cleavage site (Chen et al. 1995; Kornblatt et al. 2003) had no dramatic effect on α-cleavage.

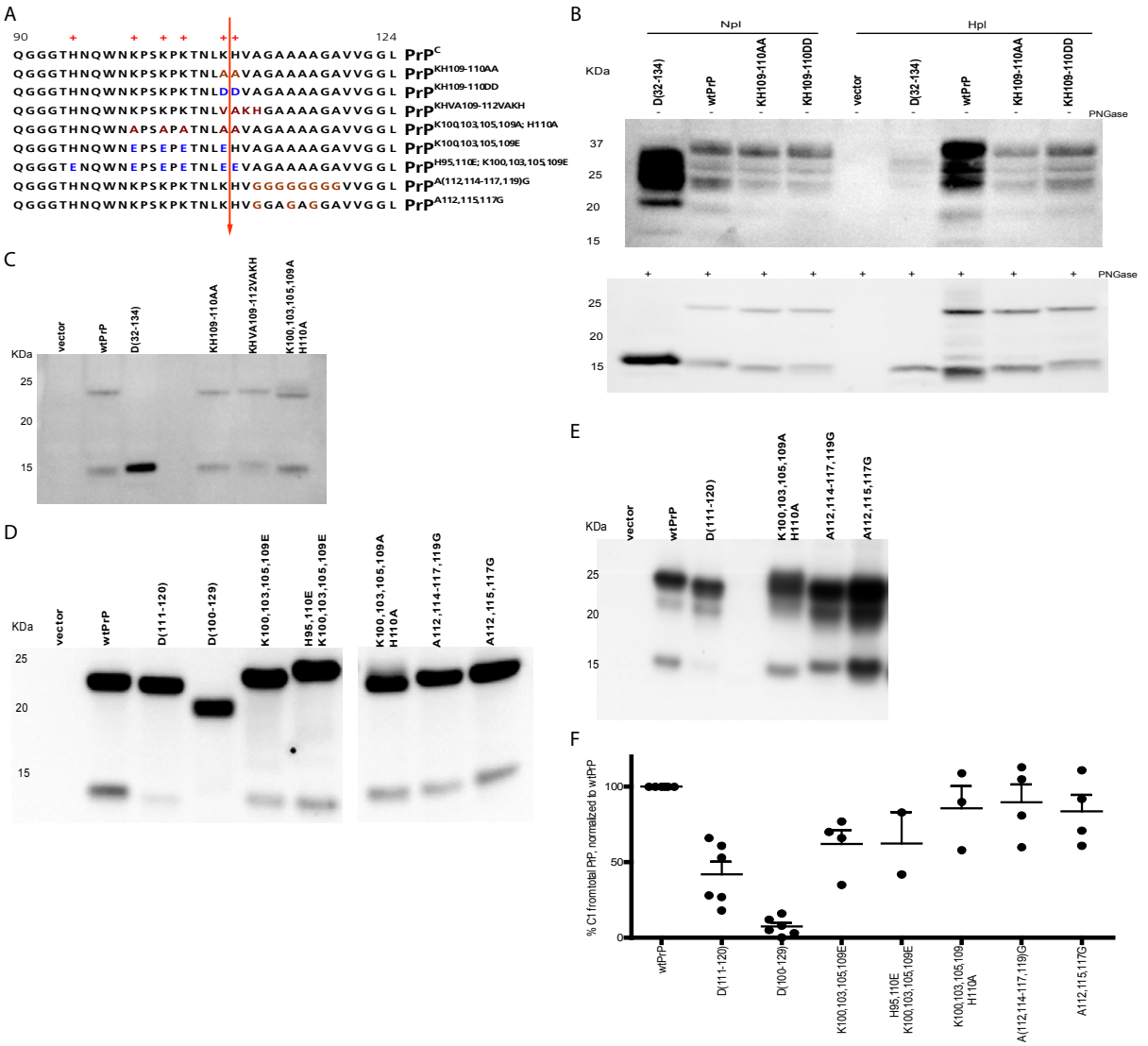


Figure 3.2: Proteolysis of PrP^C constructs with mutations in the palindrome and in the positive charges neighbouring the α-cleavage site. A) Amino-acid sequence alignment of the region (90-124), of the PrP^C constructs used. The arrow indicates the α-cleavage site. Negatively charged amino acids are represented in blue, other mutations are in red. **B)** Western blots of cell lysates of Hpl and Npl cells transfected with the various PrP^C mutants. Samples in the lower blot were PNGase treated. **C)** and **D)** Western blot of PNGase treated cell lysates of Hpl cells transfected with the various PrP^C mutants. All lanes in **D)** belong to the same blot. Detection was done with POM1. **E)** Western blot of PNGase treated cell lysates of HeLa cells transfected with various PrP^C mutants. Detection was done with POM19. **F)** Quantification of the percentage of α-cleavage of the various PrP^C mutants, based on densitometry of Western Blots. Quantifications were calculated in the linear range of the densitometric signal of independent experiments. Values refer to the amount of C1 generation comparing to total abundance of PrP^C, and are normalized to cleavage of unmutated PrP^C (wtPrP), which was assessed in the same blot. Error bars represent the SEM. In PNGase treated samples, C1 is the band at 15KDa and uncleaved PrP^C is the band at 25KDa.

3.2.3. Evaluation on the role of the PrP^C palindromic region (111-120) in α-cleavage

Given the observation of the strong independence of PrP^C proteolysis from the charged amino acids in and upstream the α-cleavage site, I decided to study the region downstream the cleavage site. In this region there is a palindrome at primary sequence level, which marks the border of the HC. This region was already mutated in a study using the N2a cell model. There, it was shown that substitution of all palindromic glycines for alanines as in the construct $PrP^{A(112,114\text{-}117,119)G}$, resulted in no or very weakly detectable C1 cleavage product (Wegner et al. 2002). Following this suggestion that exchange of alanines for glycines had a strong impact on α-cleavage, I assessed in the *Prnp*$^{-/-}$ Hpl cell model (Kuwahara et al. 1999) the cleavability of the same $PrP^{A(112,114\text{-}117,119)G}$ construct, and of $PrP^{A112,115,117G}$ which was a mutant bearing only three residue substitutions (Figure 3.2 A). In this setup, quantification of C1 generated by these palindromic mutants in Hpl cells, revealed that they were proteolytic processed in a fashion similar to the α-cleavage of PrP^C (Figure 3.2 D,F). In order to evaluate the robustness of these results, the proteolytic processing of these constructs was also assessed in the human-derived HeLa cell model. The palindromic mutants $PrP^{A(112,114\text{-}117,119)G}$, $PrP^{A112,115,117G}$ and $PrP^{\Delta(111\text{-}120)}$, and the control $PrP^{K100,103,105,109A;\ H110A}$, were transfected into HeLa cells, and the detection was done with the antibody POM19, which recognizes the globular domain of murine PrP^C but not human PrP^C (Polymenidou et al. 2008). Comparison of proteolytic processing of PrP^C and of these PrP^C mutants showed no major differences between proteolysis in HeLa cells (Figure 3.2 E), and in Hpl cells (Figure 3.2 D,F). Furthermore, $PrP^{A(112,114\text{-}117,119)G}$ and $PrP^{A112,115,117G}$ proteolysis did not appear to be strongly reduced comparing to unmutated PrP^C (Figure 3.2 E). Thence, in my setup the substitution of the palindrome downstream of the α-cleavage site, for a total or a partial poly-glycine chain, did not have a strong impact in the α-cleavage of PrP^C.

3.2.4. Evaluation of the role of hydrophobicity in PrP^C sequence in modulating α-cleavage

Another candidate biochemical feature of PrP^C sequence for modulation of α-cleavage was the high hydrophobicity neighbouring the cleavage site. My rationale was that the HC could be cell membrane associated, and that α-cleavage could depend mainly on the distance between the cleavage site and the cell membrane. This rationale implies that reduction of the hydrophobicity of the HC may impair its putative interaction with the cell membrane, and therefore decrease the rate of α-cleavage. To assess this, three PrP^C constructs were designed, bearing substitutions of hydrophobic amino acids for non-polar hydrophilic residues that had a similar size as the original ones. The small glycines (G) replaced alanines (A), and glutamines (Q) replaced the leucines (L) and valines (V) (Figure 3.3 A). The maximum amino-acid difference between each construct was four residues. The mutant $PrP^{A115\text{-}116G\ L124,129Q}$ had only a slight reduction of hydrophobicity and therefore was expected to be cleaved almost as efficiently as unmutated PrP^C (Figure 3.3 A-B). $PrP^{V120\text{-}121Q\ L124,129Q}$ had already a strong diminishment of hydrophobicity (Figure 3.3 A-B). Finally, with an almost no positive hydrophobic integral, $PrP^{A115\text{-}116G\ V120\text{-}121Q\ L124,129Q}$ was expected not to be digested if it would be confirmed the hypothesis that hydrophobicity on PrP^C sequence plays a role in modulation of α-cleavage (Figure 3.3 A-B). Quantification of C1 generation in all these three constructs showed that none of these mutations resulted in impairment on proteolysis of PrP^C (Figure 3.3 D,E). There may even have been a slight increase in proteolysis of PrP^C, but given the fact that this increase was similar within the three mutants, it suggested that the changes were independent on the hydrophobicity. Thus, reduction of hydrophobicity in the region neighbouring the α-cleavage site appears to play no significant role in the proteolytic processing of PrP^C.

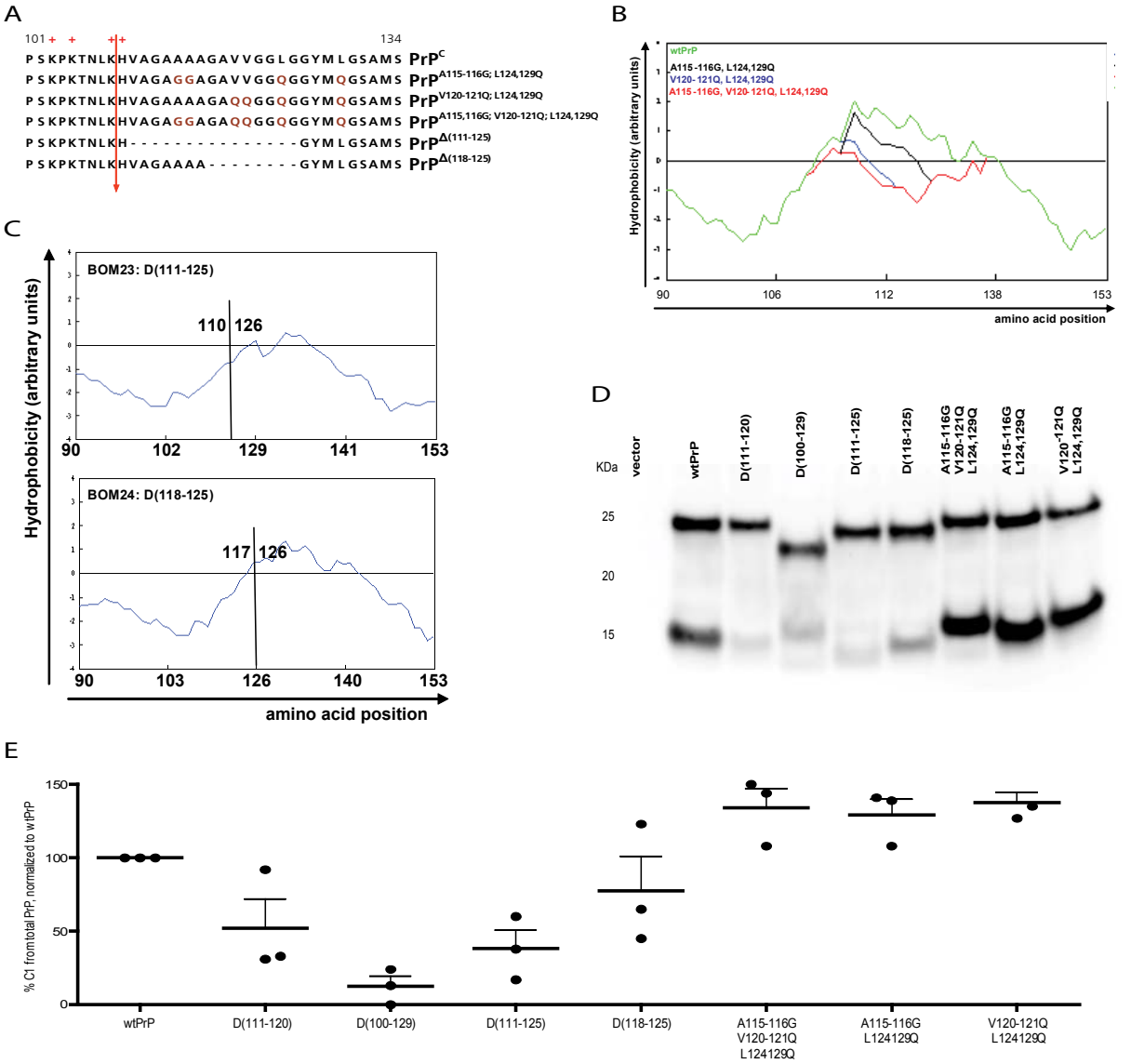


Figure 3.3: Evaluation of the role in modulating α-cleavage, of the hydrophobicity in the α-cleavage site region. A) Amino-acid sequence alignment of the region (101-134), of the PrP^{C} constructs used. The arrow indicates the α-cleavage site. Point-mutations are in red. **B)** Superimposed hydrophobicity plots of the region (90-153) of PrP^{C} in green, and the mutants $PrP^{A115\text{-}116G\ L124,129Q}$ in black, $PrP^{V120\text{-}121Q\ L124,129Q}$ in blue and $PrP^{A115\text{-}116G\ V120\text{-}121Q\ L124,129Q}$ in red. **C)** Hydrophobicity plot of the region (90-153) of $PrP^{\Delta(118\text{-}125)}$ in the upper panel and $PrP^{\Delta(111\text{-}125)}$ in the lower panel. Vertical line represents the site of the deletion, and the numbers next to it indicate the amino acid residue that flanks the deletion. **D)** Western blot of PNGase treated cell lysates of Hpl cells transfected with the various PrP^{C} deletion constructs used in the current study. Detection was done with POM1. C1 is the band around 15KDa; Uncleaved PrP^{C} is the band around 25KDa. **E)** Quantification of the percentage of α-cleavage of the various PrP^{C} mutants, based on densitometry of Western Blots. Quantifications were calculated in the linear range of the densitometric signal of independent experiments. Values refer to the amount of C1 generation comparing to total abundance of PrP^{C}, and are normalized to cleavage of unmutated PrP^{C} (wtPrP), which was assessed in the same blot. Error bars represent the SEM.

In a more extreme approach, large deletions were made in the HC, which resulted in two constructs with medium and strong reduction of hydrophobicity respectively. $PrP^{\Delta(118\text{-}125)}$ had an eight amino-acid deletion, which changed mildly the hydrophobicity around the α-cleavage site (Figure 3.3 A,C). In contrast, $PrP^{\Delta(111\text{-}125)}$

had a larger deletion that extended upstream to the beginning of the HC, which conferred a strong reduction in hydrophobicity (Figure 3.3 A,C). Measures of C1 production in these constructs (Figure 3.3 D-E) showed that α-cleavage was strongly inhibited in the construct $PrP^{\Delta(111-125)}$, which had a larger deletion and a strong decrease in hydrophobicity. In contrast, the shorter deletion $PrP^{\Delta(118-125)}$, which had a higher degree of hydrophobicity, had a milder impairment in PrP^{C} proteolysis (Figure 3.3 D-E). Therefore, using a deletion approach α-cleavage could be inhibited in mutants with low hydrophobicity in the HC. However, this inhibition could be merely dependent on the size of the deletion.

3.2.5. Assessment of the existence of a defined domain in PrP^{C} that regulates its α-cleavage

Because the strategy of inhibiting PrP^{C} proteolysis using a point-mutation approach was inefficient, and because I observed an α-cleavage impairment upon large deletions in the HC, I proceeded in this study using a deletion approach. I firstly assessed the cleavage efficiency of a construct with an almost complete deletion of the CC and the HC, $PrP^{\Delta(100-129)}$, which are the regions that flank the α-cleavage site (Figure 3.4 A). In this paradigm $PrP^{\Delta(100-129)}$, with 30 amino-acids deleted, generated virtually no C1 product in transfected Hpl cells (Figure 3.4 B,C). This suggested that there is a region within amino-acids (100-129) that is essential for modulating α-cleavage of PrP^{C}.

To further identify the region within the PrP^{C} domain (100-129) that modulates α-cleavage, the rate of C1 generation was assessed in PrP^{C} constructs with small deletions encompassing the totality of this (100-129) domain. $PrP^{\Delta(100-110)}$ and $PrP^{\Delta(105-110)}$ had a deletion in the α-cleavage site and further upstream residues (Figure 3.4 A). $PrP^{\Delta(111-120)}$ had the full palindrome downstream of the α-cleavage site deleted and $PrP^{\Delta(121-129)}$ had a deletion downstream of the palindrome (Figure 3.4 A). In respect to α-cleavage of these mutants, no differences were detected between $PrP^{\Delta(100-110)}$, $PrP^{\Delta(121-129)}$ and unmutated PrP^{C} (Figure 3.4 B-C). In contrast, $PrP^{\Delta(105-110)}$ and $PrP^{\Delta(111-120)}$ showed about 50% impairment in C1 generation (Figure 3.4 B-C). This suggests that the PrP^{C} domain (105-120), which refers to the union of the deleted domains [(105-110) + (111-120)], is important for α-cleavage of PrP^{C}. These

results are in agreement with the observation that $PrP^{\Delta(105-125)}$ does not appear to be cleaved when transgenically expressed in brain of mice (Li et al. 2007). In addition, although point mutations in the palindromic region had no remarkable influence in α-cleavage (Figure 3.3 C,F), deletion of the full palindromic region in $PrP^{\Delta(111-120)}$ resulted in a partial cleavage inhibition (Figure 3.4 B-C). Another important observation is that though $PrP^{\Delta(100-110)}$ was normally processed, the shorter deletion $PrP^{\Delta(105-110)}$ resulted in about 50% inhibition of α-cleavage. By analyzing the amino acids that compose the residue (100-104), which is present in $PrP^{\Delta(105-110)}$ and is deleted in $PrP^{\Delta(100-110)}$, it is noticeable the presence of two positively charged lysines and two prolines (Figure 3.4 A). The presence in the flank of the deletion of rigidity enhancer proline residues and of charged amino-acids may contribute to a decrease in the flexibility of the region. Such rigid structure may enhance the antagonistic effects that a deletion may have in the degree of α-cleavage. This may also apply to the other deletion that showed impairment in PrP^{C} proteolysis, $PrP^{\Delta(111-120)}$, with removal of palindromic region, which was also flanked by the charged amino-acids of the α-cleavage site (Figure 3.4 A-C).

Because PrP^{C} domain (105-120) appeared to be important for α-cleavage, I decided to identify the minimal region within this domain that would be involved in modulating the proteolytic processing of PrP^{C}. For this purpose, it was assessed the degree of C1 generation in constructs bearing deletions of different sizes, centred in the region of the α-cleavage site, and within the domain (105-120). The designed mutants, $PrP^{\Delta(106-119)}$, $PrP^{\Delta(106-114)}$, and $PrP^{\Delta(109-112)}$, had deletions of 14, 9, and 4 amino acids respectively (Figure 3.4 A). Quantification of the degree of PrP^{C} proteolysis on these constructs revealed already an impairment of about 28% of α-cleavage on the construct $PrP^{\Delta(106-119)}$, with 4 amino-acids deleted from the α-cleavage site (Figure 3.4 B-C). This impairment increased at a rate that was proportional to the increase on the size of the deletion (Figure 3.4 B-C). Plotting the α-cleavage inhibition in relation with the number of amino-acids deleted (Figure 3.4 D) revealed that α-cleavage impairment was dose dependent on the size of PrP^{C} deletion, with the deletion being centred in the region of the α-cleavage site, and being within the PrP^{C} domain (106-119). In this setup, the estimated 50% cleavage inhibition is expected to be achieved with a deletion of six amino-acids (Figure 3.4 D). The large deletion $PrP^{\Delta(100-129)}$ was used as control for total α-cleavage impairment.

Together, this suggests that there is no specific short domain in the vicinity of the PrP^C α-cleavage site that modulates the degree of proteolysis.

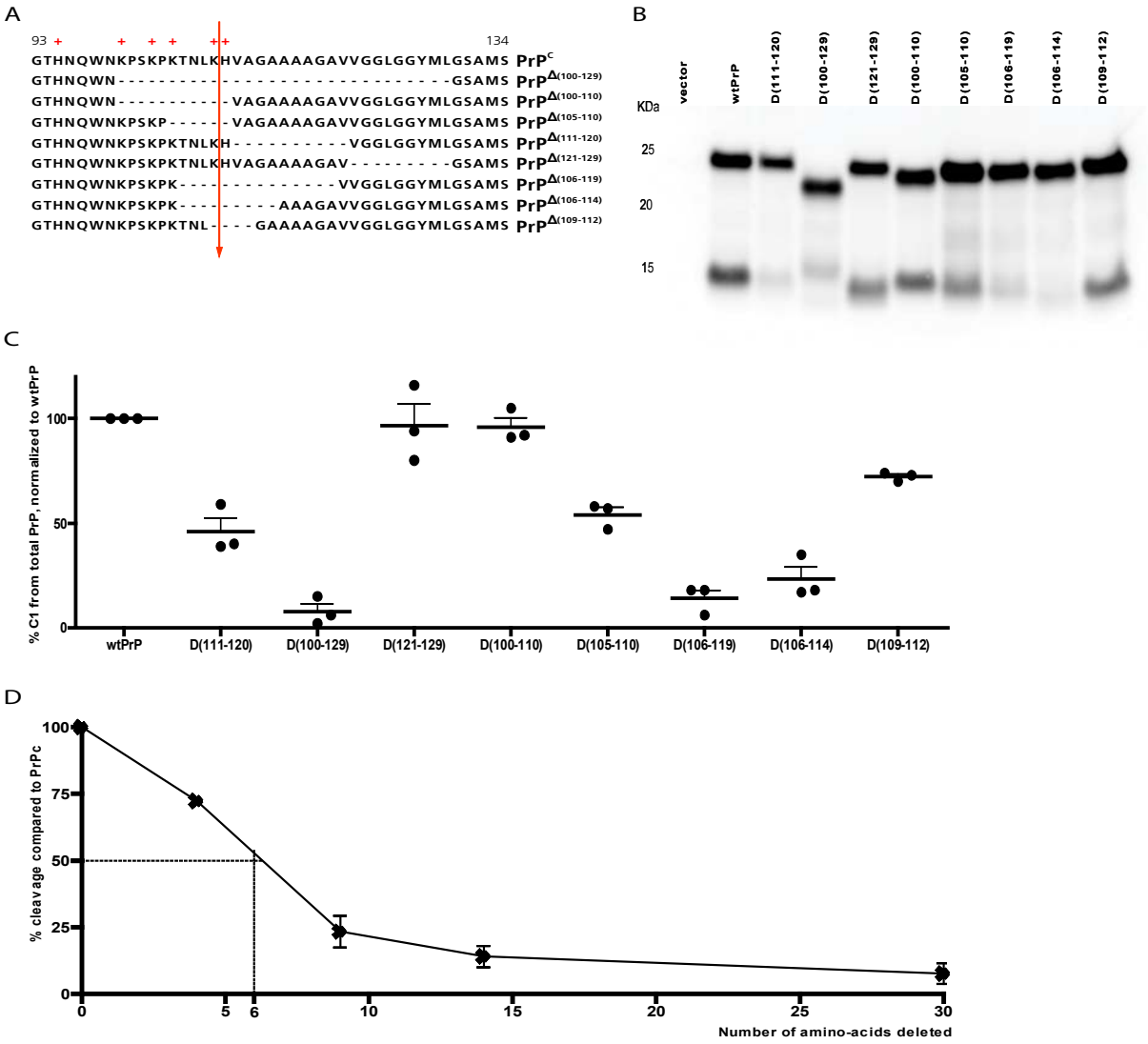


Figure 3.4: Assessment of the existence of a defined domain in PrP^C that regulates its α-cleavage. A) Amino-acid sequence alignment of the region (93-134), of the PrP^C constructs used. The arrow indicates the α-cleavage site. **B)** Western blot of PNGase treated cell lysates of Hpl cells transfected with the various PrP^C deletion constructs used in the current study. Detection was done with POM1. C1 is the band around 15KDa; Uncleaved PrP^C is the band around 25KDa. **C)** Quantification of the percentage of α-cleavage of the various PrP^C mutants, based on densitometry of Western Blots. Quantifications were calculated in the linear range of the densitometric signal of independent experiments. Values refer to the amount of C1 generation comparing to total abundance of PrP^C, and are normalized to cleavage of unmutated PrP^C (wtPrP), which was assessed in the same blot. Error bars represent the SEM. **D)** Graphic illustrating the percentage of α-cleavage impairment of PrP^C deletion mutants. Points correspond to the average value illustrated in **C)**, for the samples PrP^C, $PrP^{\Delta(109-112)}$, $PrP^{\Delta(106-114)}$, $PrP^{\Delta(106-119)}$, $PrP^{\Delta(100-129)}$, which have 0, 4, 9, 14 and 30 amino-acids deleted, respectively. In x-axis are plotted the size of the deletion of each of these constructs. Dashed line refers to the estimated deletion size that would result in 50% inhibition of α-cleavage.

3.2.6. α-cleavage in PrP molecules with PrP^{Sc}-generating point mutations

There is a model for PrP^{Sc} toxicity suggesting that an increase of hydrophobicity in the HC may result in increased generation of a transmembrane species of PrP, the ^{Ctm}PrP, which may be the toxic agent of PrP^{Sc} form (Hegde et al. 1999). This was mainly supported by a study where toxicity and PrP^{Sc} replication were assessed in a transgenic mouse model that highly overexpressed $PrP^{KH109\text{-}110II}$ in *Prnp*$^{0/0}$ background (Hegde et al. 1999). This PrP^{C} mutant had the two charged amino acids of the α-cleavage site substituted by the highly hydrophobic isoleucines. Data suggested that enhanced hydrophobicity on the PrP molecule led to higher ^{Ctm}PrP formation and increased PrP^{Sc} accumulation (Hegde et al. 1999). Here I wanted to assess if the toxicity of this mutant could be explained by inhibition of α-cleavage. For this purpose the proteolysis of $PrP^{KH109\text{-}110II}$ was assessed in the Hpl cell model. Quantification of α-cleavage suggested that this may be partially inhibited (Figure 3.5 A,C), although the values ranged from only 11% to 30% inhibition, plus one sample with 86% inhibition. This cleavage inhibition thus provides another explanation for the neurotoxicity observed in transgenic mice expressing $PrP^{KH109\text{-}110II}$ in high doses.

In order to further test the hypothesis that a slight impairment on PrP^{C} cleavage could be mildly toxic and therefore be translated into human diseases, the degree of proteolysis of constructs carrying mutations linked to human prion diseases was quantified. PrP^{P101L} is associated with Gerstmann-Sträussle-Syndrome (GSS) (Hsiao et al. 1989), PrP^{D177L} replicated Fatal Familial Insomnia (FFI) (Medori et al. 1992), and PrP^{E199K} is found in familiar CJD cases (Hsiao et al. 1991; Goldfarb et al. 1991). As control, the proteolysis of PrP^{Q218K} was assessed, which is a construct reported to be a dominant negative of PrP^{Sc} replication (Kaneko et al. 1997b). Quantification of C1 generation in Hpl cells expressing these mutants showed that PrP^{E199K}, but specially PrP^{D177N}, had inhibited PrP^{C} cleavage (Figure 3.5 B-C). Also, PrP^{P101L} showed a high variability in terms of α-cleavage rate, with low values of C1 generation observed sporadically (Figure 3.5 B-C). Finally, the PrP^{Sc} dominant negative PrP^{Q218K} was cleaved in a similar fashion as normal PrP^{C} (Figure 3.5 A,C). Together, this data suggests a link between toxicity of inherited human prion diseases, and inhibition of α-cleavage of PrP.

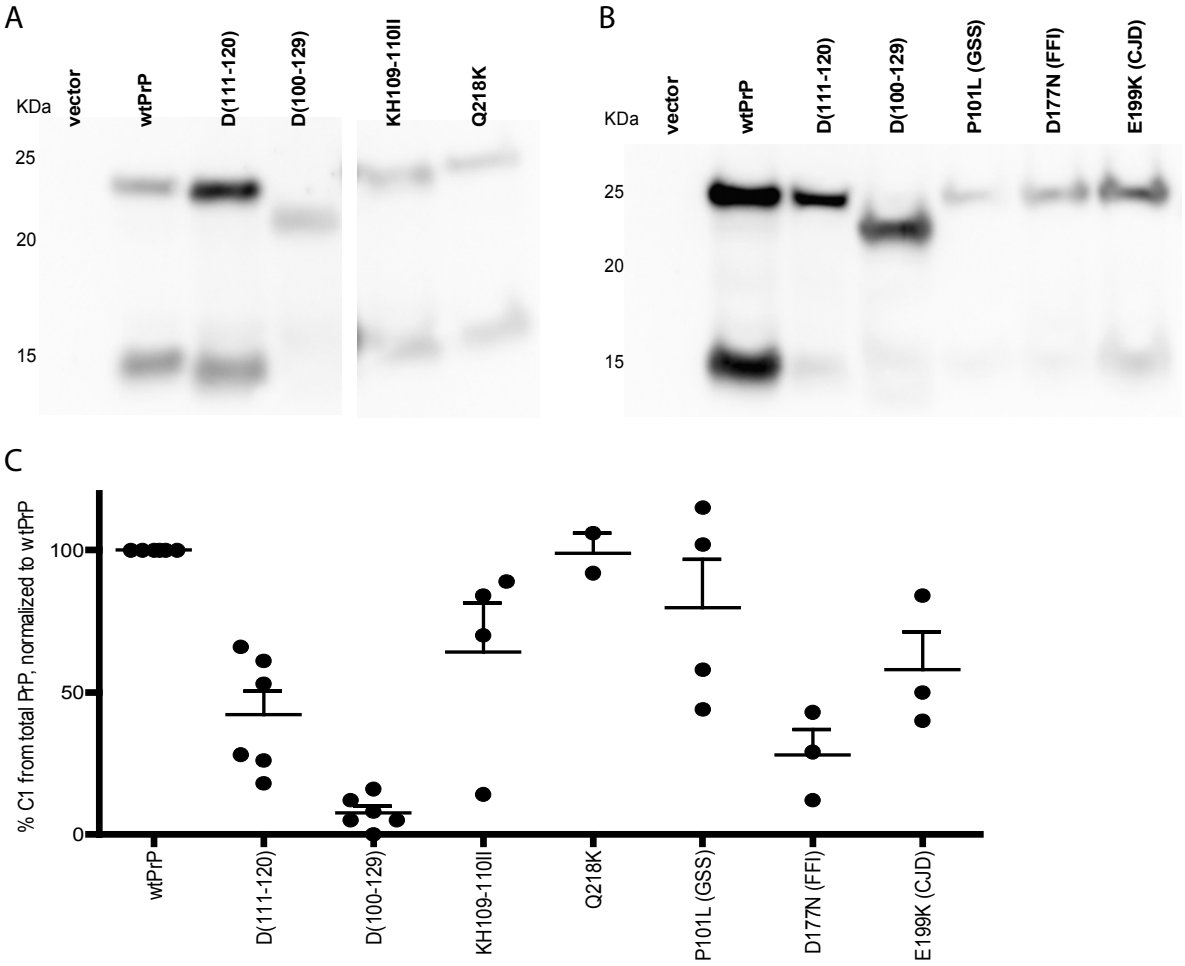


Figure 3.5: α-cleavage of PrP^C mutants with PrP^{Sc}-generating point mutations. A) and **B),** Western blots of PNGase treated cell lysates of Hpl cells transfected with various PrP^C mutants. All lanes in **A)** belong to the same blot. Detection was done with POM1. C1 is the band around 15KDa; Uncleaved PrP^C is the band around 25KDa. **C)** Quantification of the percentage of α-cleavage of the various PrP^C mutants, based on densitometry of Western Blots. Quantifications were calculated in the linear range of the densitometric signal of independent experiments. Values refer to the amount of C1 generation comparing to total abundance of PrP^C, and are normalized to cleavage of unmutated PrP^C (wtPrP), which was assessed in the same blot. Error bars represent the SEM.

3.3. Discussion

Data here suggest that PrP^C has the uncommon property of despite being proteolytic digested in the α-cleavage site, is highly independent of the sequence of the cleavage site region. Manipulations like small deletions of the cleavage site, inversion of all charges, manipulation of the hydrophobicity, alteration of domains like the palindrome, all had only partial or no effect in α-cleavage of PrP^C. However, deletion of the PrP^C domain (106-119) virtually blocked α-cleavage, while shorter deletions within this region had only a partial blocking effect. This effect increased

gradually, in function of the size of the deletion with centre in the region of the α-cleavage site.

Using the Hpl cell model system derived from the brain of *Prnp*$^{-/-}$ mice, the data in this dissertation suggested that first, substitution of positively charged residues of the α-cleavage site have no major effect on the degree of PrP^{C} proteolysis. This is in agreement with previous studies performed using GFP-fused ovine PrP^{C} mutants, in the N2a cell system (Tveit et al. 2005). Second, a complete inversion to negative, of the six positive charges of the CC, including the ones of the α-cleavage site, resulted only in a partial inhibition of C1 generation. Third, even total or partial substitution of the alanines of the palindrome that flanks the α-cleavage site, for glycines, had no or little impact in the normal proteolysis of PrP^{C}. This observation is based on the data from this dork, which was obtained in the murine Hpl and in the human HeLa cell models, and did not replicate previous findings in murine N2a cells (Wegner et al. 2002). Fourth, amino-acid substitutions that intended to decrease at different levels the degree of hydrophobicity of the region in the vicinity of the α-cleavage site also failed to reduce the proteolysis of PrP^{C}. However, an impairment of α-cleavage was observed upon the substitution of the charged residues of the cleavage site for two highly hydrophobic isoleucines. Fifth, deletions in the vicinity of the α-cleavage site, as the domains (100-110) and (121-129), had no impact in proteolysis of PrP^{C}. And deletions in the domains (105-110) and (111-120) resulted in only about 50% impairment on α-cleavage. The later result is in agreement with previous data indicating that a PrP^{C} mutant lacking the domain (114-121) had partial inhibition of α-cleavage when transgenically expressed in mice (Baumann et al. 2007). The observation that deletion of the region (100-110) had no apparent effect on the proteolysis of PrP^{C}, but the shorter deletion (105-110) partially blocked α-cleavage, can be explained by the residues in the region (100-104). These include two rigidity-conferring proline amino-acids, and two charged lysines. Thus, it suggests that prolines or charged residues flanking a deletion around the α-cleavage site may contribute for blocking the access of the PrPase to the PrP^{C} chain. Sixth, in the setup of this work α-cleavage was practically abolished only in a deletion as large as the removal of the domain (106-119) or larger portions. This reproduces the data obtained from transgenic mice expressing PrP$^{\Delta(105\text{-}125)}$, which did not appear to show C1 fragment (Li et al. 2007). However, shorter deletions within the PrP^{C} domain (106-119) and centred in the α-cleavage site region appeared to only partially block

proteolysis of PrP^C, in a blockage dependent manner of the size of the deletion. A 50% blockage of α-cleavage was estimated to be obtained with a six amino-acid deletion. Therefore, I can conclude that α-cleavage of PrP^C has a strong plasticity concerning the amino-acid sequence and the physical and structural patterns on the vicinity regions of the α-cleavage site. This strong plasticity may correlate with the fact that all reports that refer to transgenic mice expressing a PrP^C mutant that had a blockage of α-cleavage, have always shown neurotoxicity (Baumann et al. 2007; Shmerling et al. 1998; Li et al. 2007). If this correlation holds true, it would be expectable that α-cleavage of PrP^C would be a process with strong plasticity, because its inhibition could result in toxicity. Finally, the cleavage of part of the mutants used in this study was assessed using other biological systems, as *Prnp*$^{-/-}$ mouse derived Npl cells (Nishimura et al. 2007), human HeLa cells, and mouse embryonic fibroblasts. Together the data suggested that the patterns of α-cleavage of PrP^C mutants are comparable between the Hpl cell model, and the other biological systems addressed.

The strong plasticity of PrP^C α-cleavage can possibly be explained by a high level of redundancy in terms of PrPases involved in PrP^C cleavage. Alternatively, there are proteases that partially replicate the high cleavage plasticity of the putative PrPase. One example is the β-amyloid precursor protein (APP) -cleaving γ-secretase. In this respect it has been reported that point mutations in APP have the property of shifting the amino acid cleavage site, but often do not block the generation of APP cleaved fragment (McCarthy et al. 2009; Qi-Takahara et al. 2005; Zhao et al. 2005). γ-secretase cleaves within a transmembrane region, whose length has been suggested to be determinant for definition of the cleavage site (McCarthy et al. 2009). As for PrP^C, the α-cleavage site is in the interface between a charged and a hydrophobic domain, and it is still not resolved if this later region can associate with the lipidic bilayer. Therefore, though the proteolytic features of PrP^C are very uncommon, there are other examples of proteins with a high plasticity at the level of the cleavage site sequence.

In respect to α-cleavage associated with mutations that replicate familial prion diseases, there appears to be an impairment of the proteolytic processing of these constructs. In addition, α-cleavage appeared to also be impaired in $PrP^{KH109\text{-}110II}$, which is a mutant reported to enhance PrP^{Sc} replication and to be toxic in transgenic mice (Hegde et al. 1999). And in the construct PrP^{Q218K} construct, which dominantly

inhibits PrP^{Sc} replication (Kaneko et al. 1997b), the degree of α-cleavage was similar to the one detectable in PrP^{C}. If this PrP^{C} cleavage inhibition is on the basis of the toxicity of these mutants, or if it is a consequence of it, remains to be established.

Together, in this study testing the role on α-cleavage of diverse biochemical properties in PrP^{C} sequence, it is shown that α-cleavage is highly independent of the primary and secondary structure in the vicinity of the α-cleavage site. It is also shown that the PrP^{C} domain (106-119) is important for modulating PrP^{C} proteolysis and that inhibition of this proteolysis was dependent on the number of residues removed from this domain.

3.4. Outlook

This study provided a very clear vision about the role in α-cleavage of the domains in PrP^{C} sequence that are in the neighbouring regions of this proteolytic site. However, there are few issues in this domain that remain to be clarified. One of the questions is the evaluation of the role for this cleavage that the rigidity of the PrP N-proximal flexible sequence has. The main reason for this is the observation that the presence of rigidity-conferring proline residues in the flanks of a deletion can be correlated with the inhibition of α-cleavage.

Another important point that remains to be evaluated is the cleavability of the PrP^{C} constructs with deleted globular domain. I showed that contrary to Hpl, Npl and NIH-3T3, in N2a-PK1 it is possible to detect N1 cleavage product, but the N1 generation of these constructs was only assessed in the Hpl cell model, which does not express PrP^{C}. It remains to evaluate the cleavage of these constructs using a model system where N1 detectable. Moreover, it would be interesting to evaluate the biological significance of the two particularities of the N2a-PK1 cell line, which are PrP^{Sc} replication and N1 detectability.

But the most important piece of evidence that has been missing is the direct connection between the α-cleavage of PrP^{C} and cell toxicity. It has recently been shown an assay that may easily allow the measurement of toxicity of several PrP^{C} mutants (Rambold et al. 2008), however this data remains to be confirmed.

The other important question in the field is the identification of the PrPase. Though many players have been suggested (Jimenez-Huete et al. 1998; Cisse et al. 2005;

Vincent et al. 2001; Shyng et al. 1993; Kornblatt et al. 2003; Vincent et al. 2000; Laffont-Proust et al. 2005; Yadavalli et al. 2004), the results remain contradictory and the consensus is not yet established. The interaction between the PrPase candidates and these mutants could be assessed, in order to identify the interaction site in PrP^C sequence. In another way, proteomic studies urge to be performed, in order to identify proteases or other interactors that associate differently between PrP^C and PrP^C mutants with cleavage impairment.

There has also been published a fluorimetric assay that should allow the assess α-cleavage at large scale (Cisse et al. 2006). Though it is still not completely established the degree of which this technique replicates the data originated using traditional methods, it is unquestionable that this is a good tool for PrP^C cleavage field. It would be interest to do genetic studies of pooled cell clones that exhibit a high degree of PrP^C cleavage, and other ones that have this process impaired. Also, it would be interesting to do a similar genetic study to identify the special characteristics of N2a-PK1 cells within this feature of conserving an intact N1.

Many other interesting research projects may be raised from this and other reports, which would allow to address the most important open questions about α-cleavage, and consequently to help solving the big mystery about the physiological function of PrP^C and the mechanisms of PrP toxicity. Here we aimed to suggest the experiments that we considered more pertinent in the field of α-cleavage.

3.5. Materials and methods

3.5.1. Cloning

Murine PrP^C was amplified from total brain cDNA, using the primers SY6 and SY7 (Table 1), which introduces the *BamH*I and the *Sal*I cleavage sites, respectively. The PCR products were digested with *BamH*I and *Sal*I and the targeted vector, pBMN-I-EGFP (Addgene, Plasmid 1736), was digested with *BamH*I and *Xho*I. Fragments were later purified from an agarose gel, using the Amersham GFX PCR DNA and Gel Band Purification Kit. The PCR products and the open plasmid were ligated using the Roche Rapid DNA Ligation Kit. The products were once again purified using Amersham GFX PCR DNA and Gel Band Purification Kit, and transformed into

TOP10 competent *E. coli* (Invitrogen). Other constructs were performed in two PCR steps using the primers and templates listed in Table1. In the first step, PCR was performed with 1) SY6 (Table1) and the reverse plasmid referred in Table1; 2) SY7 (Table1) and the forward plasmid referred in Table1. Second step was performed with a PCR using 1uL of PCR product 1) and 2), and using SY6 and SY7 to make the full construct. PCR product purification, digestion and ligation were done in a similar way as described for PrP^{C}. Constructs $PrP^{\Delta(118-125)}$ and $PrP^{\Delta(111-125)}$ were done using the QuikChange kit (Stratagene), using the primers indicated in Table 1. Dv1 construct originates from previous experiments (Ott et al. 2008).

Table 1: List of the primers used in this study.

Primer name	sequence 5' ---> 3'	direction	Description	Code	Template
A5	aaa cca aaa acc aac ctc gat gat gtg gca ggg gct gcg gca	Fw	PrP KH109-110DD	BOM3	PrPc
A6	tgc cgc agc ccc tgc cac atc atc gag gtt ggt ttt tgg ttt	Rv	PrP KH109-110DD	BOM3	PrPc
A11	aaa cca aaa acc aac ctc gcc gcc gtg gca ggg gct gcg gca	Fw	PrP KH109-110AA	BOM6	PrPc
A12	tgc cgc agc ccc tgc cac ggc ggc gag gtt ggt ttt tgg ttt	Rv	PrP KH109-110AA	BOM6	PrPc
A13	aaa cca aaa acc aac ctc gtg gca aag cat ggg gct gcg gca	Fw	PrP KHVA109-112VAKH	BOM7	PrPc
A14	tgc cgc agc ccc atg ctt tgc cac gag gtt ggt ttt tgg ttt	Rv	PrP KHVA109-112VAKH	BOM7	PrPc
A15	ggg ggt acc cat aat cag tgg aac gcc ccc agc gca cca gca acc aac ctc gcc gcc gtg gca ggg gc	Fw	PrP K100,103,105,109A H110A	BOM8	BOM6
A16	gc ccc tgc cac ggc ggc gag gtt ggt tgc tgg tgc gct ggg ggc gtt cca ctg att atg ggt acc ccc	Rv	PrP K100,103,105,109A H110A	BOM8	BOM6
A17	aaa acc aac ctc aag cat gtg gga ggg ggt ggg gga ggt ggg gga gta gtg ggg ggc ctt ggt	Fw	PrP A112,114-117,119G	BOM9	PrPc
A18	acc aag gcc ccc cac tac tcc ccc acc tcc ccc acc ccc tcc cac atg ctt gag gtt ggt ttt	Rv	PrP A112,114-117,119G	BOM9	PrPc
A21	aaa acc aac ctc aag cat gtg gga ggg gct ggg gca ggt ggg gca gta gtg ggg ggc ctt	Fw	PrP A112,115,117G	BOM11	PrPc
A22	aag gcc ccc cac tac tgc ccc acc tgc ccc agc ccc tcc cac atg ctt gag gtt ggt ttt	Rv	PrP A112,115,117G	BOM11	PrPc
A23	gtg gca ggg gct gcg gca gct	Fw	PrP D(100-110)	BOM12	PrPc
A24	ccc tgc cac gtt cca ctg att atg ggt acc ccc t	Rv	PrP D(100-110)	BOM12	PrPc
A23	gtg gca ggg gct gcg gca gct	Fw	PrP D(105-110)	BOM13	PrPc
A26	ccc tgc cac tgg ttt gct ggg ctt gtt cca ctg att	Rv	PrP D(105-110)	BOM13	PrPc
A27	ggg agc gcc atg agc agg ccc at	Fw	PrP D(121-129)	BOM14	PrPc
A28	ggc gct ccc tac tgc ccc agc tgc cgc agc ccc t	Rv	PrP D(121-129)	BOM14	PrPc
A29	gc aaa cca aaa gca gct ggg gca gta gtg ggg ggc ctt	Fw	PrP D(106-114)	BOM15	PrPc
A30	ttt tgg ttt gct ggg ctt gtt	Rv	PrP D(106-114)	BOM15	PrPc
A31b	aa acc aac ctc gct gcg gca gct ggg gca gta	Fw	PrP D(109-112)	BOM16	PrPc
A32	gag gtt ggt ttt tgg ttt gct	Rv	PrP D(109-112)	BOM16	PrPc
A34	gc aaa cca aaa gta gtg ggg ggc ctt ggt ggc ta	Fw	PrP D(106-119)	BOM17	PrPc
A30	ttt tgg ttt gct ggg ctt gtt	Rv	PrP D(106-119)	BOM17	PrPc
B14	aaa acc aac ctc aag cat ¦ ggc tac atg ctg ggg agc	Fw	PrP D(111-125)	BOM23	PrPc
B15	gct ccc cag cat gta gcc ¦ atg ctt gag gtt ggt ttt	Rv	PrP D(111-125)	BOM23	PrPc
B16	gca ggg gct gcg gca gct ¦ ggc tac atg ctg ggg agc	Fw	PrP D(118-125)	BOM24	PrPc
B17	gct ccc cag cat gta gcc ¦ agc tgc cgc agc ccc tgc	Rv	PrP D(118-125)	BOM24	PrPc
B18	aag cat gtg gca ggg gct ggg gga gct ggg gca caa cag ggg ggc cag ggt ggc tac atg cag ggg agc gcc atg agc agg	Fw	PrP A115-116G V120-121Q L124,129Q	BOM25	PrPc
B19	cct gct cat ggc gct ccc ctg cat gta gcc acc ctg gcc ccc ctg ttg tgc ccc agc tcc ccc agc ccc tgc cac atg ctt	Rv	PrP A115-116G V120-121Q L124,129Q	BOM25	PrPc
B20	aag cat gtg gca ggg gct ggg gga gct ggg gca gta gtg ggg ggc cag ggt ggc tac atg cag ggg agc gcc atg agc agg	Fw	PrP A115-116G L124,129Q	BOM26	PrPc
B21	cct gct cat ggc gct ccc ctg cat gta gcc acc ctg gcc ccc cac tac tgc ccc agc tcc ccc agc ccc tgc cac atg ctt	Rv	PrP A115-116G L124,129Q	BOM26	PrPc
B22	gct gcg gca gct ggg gca caa cag ggg ggc cag ggt ggc tac atg cag ggg agc gcc atg agc agg	Fw	PrP V120-121Q L124,129Q	BOM27	PrPc

Primer name	sequence 5' ---> 3'	direction	Description	Code	Template
B23	cct gct cat ggc gct ccc ctg cat gta gcc acc ctg gcc ccc ctg ttg tgc ccc agc tgc cgc agc	Rv	PrP V120-121Q L124,129Q	BOM27	PrPc
BAUF95	t cag tgg aac gag ccc agc gaa cca gaa acc aac ctc gag cat gtg gc	Fw	PrP K100,103,105,109E	FBOM1	PrPc
BAUF96	gc cac atg ctc gag gtt ggt ttc tgg ttc gct ggg ctc gtt cca ctg a	Rv	PrP K100,103,105,109E	FBOM1	PrPc
BAUF97	ga ggg ggt acc gag aat cag tgg aac gag ccc agc gaa cca aaa ac	Fw	PrP H95E K100,103E	FBOM2	PrPc
BAUF98	gt ttt tgg ttc gct ggg ctc gtt cca ctg att ctc ggt acc ccc tc	Rv	PrP H95E K100,103E	FBOM2	PrPc
BAUF99	ccc agc gaa cca gaa acc aac ctc gag gag gtg gca ggg g	Fw	PrP H95,110E K100,103,105,109E	FBOM3	FBOM2
BAUF100	c ccc tgc cac ctc ctc gag gtt ggt ttc tgg ttc gct ggg	Rv	PrP H95,110E K100,103,105,109E	FBOM3	FBOM2
DOM2f	gtg cgt cac cca gta caa gaa gga gtc cca ggc	Fw	PrP Q218K	Dv1	PrPc
DOM2r	gcc tgg gac tcc ttc ttg tac tgg gtg acg cac	Rv	PrP Q218K	Dv1	PrPc
Bauf5	gca aac caa aaa cca acc tca tca tcg tgg cag ggg ctg cgg cag	Fw	PrP KH109-110II	C12	PrPc
Bauf6	ctg ccg cag ccc ctg cca cga tga tga ggt tgg ttt ttg gtt tgc	Rv	PrP KH109-110II	C12	PrPc
SY71	cag tgg aac aag ctc agc aaa cca aaa	Fw	PrP P101L	C32	PrPc
SY72	ttt tgg ttt gct gag ctt gtt cca ctg	Rv	PrP P101L	C32	PrPc
SY73	aac ttc gtg cac aac tgc gtc aat atc	Fw	PrP D177N	C33	PrPc
SY74	gat att gac gca gtt gtg cac gaa gtt	Rv	PrP D177N	C33	PrPc
SY75	gag aac ttc acc aag acc gat gtg aag	Fw	PrP E199K	C34	PrPc
SY76	ctt cac atc ggt ctt ggt gaa gtt ctc	Rv	PrP E199K	C34	PrPc
SY6	cgc gga tcc aat tta gga gag cca agc aga				
SY7	acg cgt cga cca cga gaa tgc gaa gga aca				

3.5.2. Analysis of the constructs

Most of the work was performed in Hpl cells (Kuwahara et al. 1999). Other cell lines used were N2a-Pk1 (Klohn et al. 2003), NIH-3T3 (Todaro and Green 1963), Npl (Nishimura et al. 2007), Hela (Gey et al. 1952) and primary embryonic fibroblasts obtained from E12,5 *Prnp*$^{-/-}$ embryos.

Transfection was performed using Lipofectamine (Invitrogen) and Plus Reagent (Invitrogen), into 80% confluent cells. Cells were harvested using 10mM EDTA, 2 days after transfection. Cell lysis was performed on ice for 1h, using 1% Triton-X-100 and 1% NP-40, in Tris buffer at pH7.5, with NaCl and protease inhibitors (Complete; Roche). Lysate supernatant was collected after centrifugation for 30 min at 16000g at 4°C. Supernatant was then treated with PNGase (NEB), and loaded onto 12% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by wet blotting. Primary antibody was anti PrP^{C} POM1 (Polymenidou et al. 2008), and POM19 (Polymenidou et al. 2008) in case of human derived Hela cells, diluted 1:10'000 from a 1ug/ul stock. Peroxidise-labelled anti-mouse IgG1 diluted 1:10'000 (Zymed) was used as secondary antibody. Antibodies were probed for 1h at room temperature or over-night at 4°C in a 1% Top-Block solution (FLUKA) in PBS-Tween 20. For membrane blocking, a 5% Top-Block solution was used. Development was done using ECL detection system (Pierce).

Densitometry of the PrP bands was done using TINA v2.09g (raytest Isotopenmessgeräte), in the linear range of the band intensity.

3.5.3. Hydrophobicity plots

Hydrophobicity plots were calculated for using DNAMAN software (Lynnon BioSoft, Canada), for window intervals of nine residues.

4. The physiological function of PrP^C in the immune system

4.1. Introduction

4.1.1. Aim of the study

As discussed in section 2, one of the crucial assumptions in the model proposed in this dissertation is that PrP contributes for cell activation, and that miss-regulation of this cell activation can lead to cell death. As refereed, though there are many studies that support this statement (Aguzzi et al. 2008a; Hu et al. 2008; Zomosa-Signoret et al. 2008; Mazzoni et al. 2005), this question is still far from being resolved. Therefore, I decided to put efforts in evaluating if modulation of cell activation is a feature of the physiological function of PrP^C.

Lymphocytes are very intensely studied cells whose development includes positive and negative selection steps which are important to eliminate hypo- and hyper-responsive cells respectively. These steps are well described and are crucial to assure that the individual is not immunodeficient, in case of hypo-responsiveness, nor allergic, in case of hyper-responsiveness. Given these characteristics, and if the assumption aforedescribed is correct, it is expectable that manipulation of PrP^C expression in different lymphocytes would lead to altered threshold of cell activation, and therefore would induce or inhibit apoptosis in different developmental stages. The final deduction is that abnormal PrP^C expression profiles should result in detectable changes in lymphocyte homeostasis.

For this purpose, I decided to analyse the frequencies of various lymphocyte populations in mice that transgenically expressed PrP^C in different doses and in distinct cellular subsets, and to assess the effect of PrP^C expression in lymphocyte homeostasis. The working hypothesis was that PrP^C overexpressing mice would have diminished cell populations that were more sensitive to over-activation. Also, I expected to find a decreased expression of costimulatory molecules involved in the cellular activation pathways, in order to compensate for transgenic PrP^C overexpression.

4.1.2. Brief overview about murine B-cell development

B-cells development starts with the migration to the bone marrow of multipotent lymphoid precursors (CLP), which derived form hematopoietic stem cells (HSC). In bone marrow, they start expressing B220, also named CD45R, together with low levels of the heat stable antigen (HSA) also called CD24, and CD43, and are named pre-pro-B-cells (Figure 4.1), or Fraction A, according to Hardy's nomenclature (Hardy et al. 1991; Hardy 2003). The next stage is Fraction B, or early pro-B-cell, when the immunoglobulin (Ig) heavy chain D-J rearrangements occur (Hardy et al. 1991). Here, cells express at the surface high levels of CD24 and CD19. This is followed by heavy chain V-DJ rearrangement and membrane expression of BP-1 and the pre-BCR (pre B-cell receptor) Igα-Igβ heterodimer in association with calnexin (Nagata et al. 1997), at Fraction C or late pro-B-cell stage. During this pro-B-cell stages, progression in development depends mainly on activation of transcription factors like EBF (Lin and Grosschedl 1995), E12 and E47 (Bain et al. 1994), and on extrinsic factors like IL7-R signalling (Miller et al. 2002), and are fairly independent of pre-BCR signalling (Hagman and Lukin 2006). But to advance to the pre-B-cell phase, cell need to successfully pass the pro- to pre-B-cell checkpoint which depends mainly on pre-BCR efficient signalling (Kitamura et al. 1991; Wang and Clark 2003). The pre-B-cell stage proceeds with an increase in cell size, followed by 2 to 5 mitotic cycles (Rolink et al. 2000) and an augment of IL-7 responsiveness (Marshall et al. 1998). The step that follows is the small pre-B cell stage, where light chain Ig rearrangements initiate, and CD24, CD43 and IL-7R are downreagulated (Hardy et al. 1991). The successful rearrangement of the light chain, and is detected by de membrane deposition of newly formed IgM, together with the CD79 complex (CD79a, also termed Igα, associated with CD79b, also known as Igβ). It fallows a strict process of negative selection, which eliminates by anergy the cells that bind with low avidity to the antigen (Fulcher and Basten 1994), and by deletion the autoreactive ones (Erikson et al. 1991; Hartley et al. 1991). These last high affinity antigen binding cells can also undergo secondary Ig gene rearrangements, in order to produce a less autoreactive receptor and escape deletion (Gay et al. 1993; Tiegs et al. 1993). The cells that manage to be approved in all these checkpoints maturate and leave the bone marrow. Although there are many possible paths that can follow, the selected cells eventually enter the splenic red pulp, as T1 transitional cells. For the transition

from immature B-cell to T1 it is crucial the presence of an activation signal mediated by CD79b molecule (Reichlin et al. 2001). These lymphocytes start expressing the FcεRII CD23 molecule and the complement receptor CR2, also known as CD21, together with the high expression of IgD in the membrane, by alternative RNA splicing, in conjunction with high IgM, which move them to the T2 transitional category (Loder et al. 1999; Wang and Clark 2003). In a Burton's tyrosine kinase (Btk) dependent manner, T2 cells need to get enough B-cell receptor (BCR) -antigen signalling in order to proceed to the Follicular (FO) B-cell stage (Fang et al. 1998), where IgM is downregulated, and the cell can enter into the germinal centres and give rise do plasma and memory cells. Alternatively, if the BCR-antigen signal is weaker, B-cells move to the Marginal Zone (MZ) of the splenic follicules, maintain high IgM expression and decrease IgD, and turn into MZ B-cells (Cariappa et al. 2001).

Other important cell populations are the B1a and B1b cells. These are long-lived self-renewing B-cells, located mainly in pleural and the peritoneal cavities, which are maintained or selected by high avidity interaction with self antigens (Hayakawa et al. 1999). B1a cells develop from the fetal liver and therefore lack the Terminal Deoxynucleotidyl Transferase (TdT) DNA polymerase which is important for the DNA VDJ recombination of Ig heavy chain. In contrast B1b, which are a very low fraction of the B1 cells and yet, are originated by bone-marrow derived stem cells and phonotypically are distinguished by B1a cells, by the absence of CD5, a negative regulator of lymphocyte activation (Tarakhovsky et al. 1995; Bikah et al. 1996). These B1 B-cells are the major source of secretion of natural antibodies and depend on a strong TCR-antigen signalling to develop or to be maintained (Pillai et al. 2004).

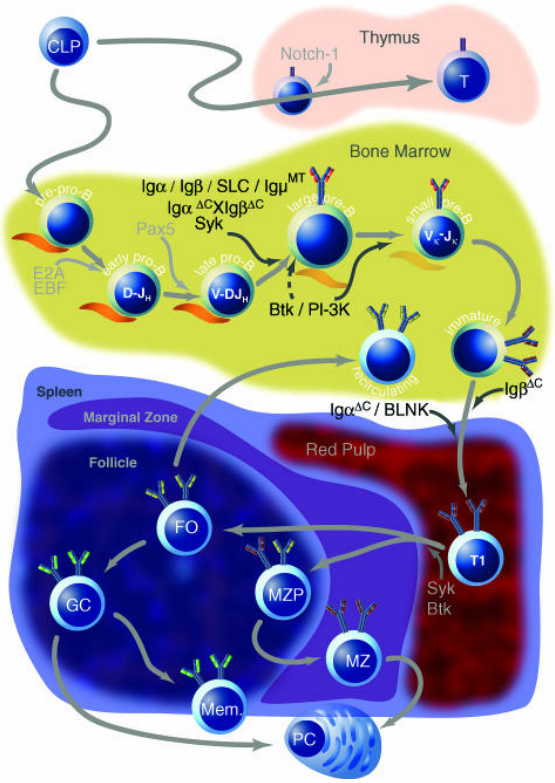


Figure 4.1: B-cell development. CLP – Common Lymphoid Precursor; T1 – Transitional 1 immature B-cells; MZ – Marginal Zone B-cell; MZP – Progenitor of Marginal zone cell; FO – Follicular B-cell; GC – Germinal Centre B-cell; Mem. – Memory cell; PC – Plasma cell. From (Wang and Clark 2003).

4.1.3. Brief overview about murine T-cell development

In adult mice, bone marrow hematopoietic stem cells (HSC) give rise to $Lin^{-}Thy1.1^{-}Sca1^{+}c\text{-}kit^{+}CD62L^{+}$ precursors, known as LSK-$CD62L^{+}$ (Perry et al. 2003; Perry et al. 2004), which develop to early lymphoid precursors (ELP) (Medina et al. 2001) that can originate the common lymphoid precursors (CLP) (Kondo et al. 1997). Excluding this later population, the first two mentioned ones can also be found in the blood, and are thought to be the progenitors of the early T lineage progenitors (ETP) that are found in the thymus (Schwarz and Bhandoola 2004). These ETP cells (Borghesi et al. 2004) may correspond to the DN1a and DN1b populations (Porritt et al. 2004) that are $CD44^{+}CD25^{-}c\text{-}kit^{hi}CD24^{+}$. In an earlier stage they are $Flt3^{+}$, and later switch off this marker (Sambandam et al. 2005), before they deposit at the membrane the α chain of IL-2 receptor named CD25 and advance to the stage DN2 (Figure 4.2). After this, cells switch off CD44 and move to the DN3 developmental phase. In the period cells spend as DN2 and DN3, cells migrate to the thymic cortex in a CCR7-dependent process (Witt and Robey 2004; Misslitz et al. 2004), and rearrange the

gene that encodes the β chain of the T-cell receptor (TCRβ). This protein is then expressed in the membrane in a complex with an invariant pre-Tα chain, which globally constitutes the pre-TCR. At this stage there is a checkpoint for the correct functionality of the newly formed pre-TCR, where cells that do not receive efficient pre-TCR signals undergo apoptosis (Newton et al. 2000; Mandal et al. 2005). After this checkpoint there is the down-regulation of CD25 (DN4 stage), and the switch on of CD4 and CD8 expression, accompanied with intense proliferation. The resulting $CD4^+CD8^+$ cells (DP stage) stop dividing and start rearranging the TCRα, originating a mature TCRαβ complex (Figure 4.2). At this stage cells face the second important checkpoint, where a functional TCRαβ is positively selected and a hyper-reactive or low-reactive one is eliminated (Goldrath and Bevan 1999; Starr et al. 2003). Curiously, this selection process takes into account the community effect (Gurdon 1988), because the presence of cells with non-functional TCR favours the positive selection of cells with a functional one (Canelles et al. 2003). Also in this DP phase, cells interact with antigen presented by the Major Histocompatibility Complex class I (MHC I) or MHC II, and depending of the class of MHC that provided the optimal surviving signal, the expression of either CD4 or CD8 respectively is switched off, resulting in CD8 SP (single positive) or CD4 SP cells. From the positively selected CD4 SP cells, it appears that the fraction of the ones with higher reactivity to the presented antigen (Ag) complexed MHC II will end up turning into regulatory T-cells (Jordan et al. 2001; Apostolou et al. 2002) which are $CD4^+CD25^+Foxp3^+$ and play an important role in suppressing the activation of naïve T-cells (Sakaguchi et al. 1995). These SP cells will down-regulate CD24, and up-regulate CCR7 (Rosen et al. 2003), a chemokine receptor that is important for migration from the thymic cortex, go back to the medulla (Ueno et al. 2004) and out of the thymus.

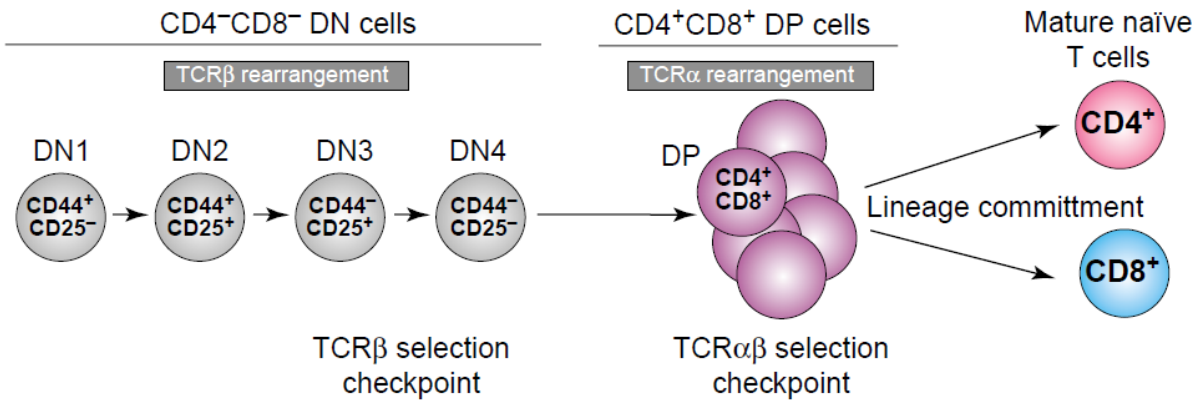


Figure 4.2: Representation of T-cell development in Thymus. (Zamoyska and Lovatt 2004)

Another set of T-cells are the γδT-cells, which are a population mainly located in the peritoneal cavity and in the gut mucosa, and that react to various Ag independently of its association with MHC. These cells are distinguished from the αβT-cells by having a TCR complex composed by a γ and a δ chain, and not the more common α and β. The development divergence appears to occur in the DN2 and DN3 developmental stages (Ciofani et al. 2006), where it has been suggested that negatively selected cells bearing γδTCR may convert into the αβTCR lineage (Haks et al. 2005; Hayes et al. 2005), which appears to demand weaker signals for being positively selected (Irving et al. 1998; Yamasaki et al. 2006).

4.1.4. Previous studies about function of PrP^C in lymphocyte homeostasis

One of the pioneering studies at the level of assessing the function of PrP^C in the immune system was performed by Cashman and colleagues (Cashman et al. 1990) where they showed that lymphocyte activation induces increased PrP^C expression. Other studies followed which confirmed this observation on activated T-cells (Mabbott et al. 1997; Bainbridge and Walker 2005), and showed that this could occur within 4-8h of in vitro primary culture with Con A (Kubosaki et al. 2003). Another important study showed that PrP^C on stimulating dendritic cells (DC), but not on T-cells, is important for efficient T-cell activation (Ballerini et al. 2006). Also at lymphocyte development level it was suggested that PrP^C is important for the activity of long term hematopoietic stem cell in bone marrow (Zhang et al. 2006). At another level, Marche and colleagues studied the T-cell development in thymus of mice over-expressing PrP^C and observed a partial arrest in the DN3 phase and a strong Cu^{2+}-dependent decrease of the DP and SP populations when PrP^C was highly over-expressed (Jouvin-Marche et al. 2006). One intriguing factor on these studies is the observation that PrP^C expression profiles in immune system differ in a high degree between species (Linden et al. 2008), therefore advising for some caution when extrapolating results. In addition, the data from Zabel and colleagues showing that many putative effects of PrP^C can be explained by a transgene insertion artifact in

one PrP^C overexpressing mouse line adverts for prudence in interpreting results from experiments with transgenic animals.

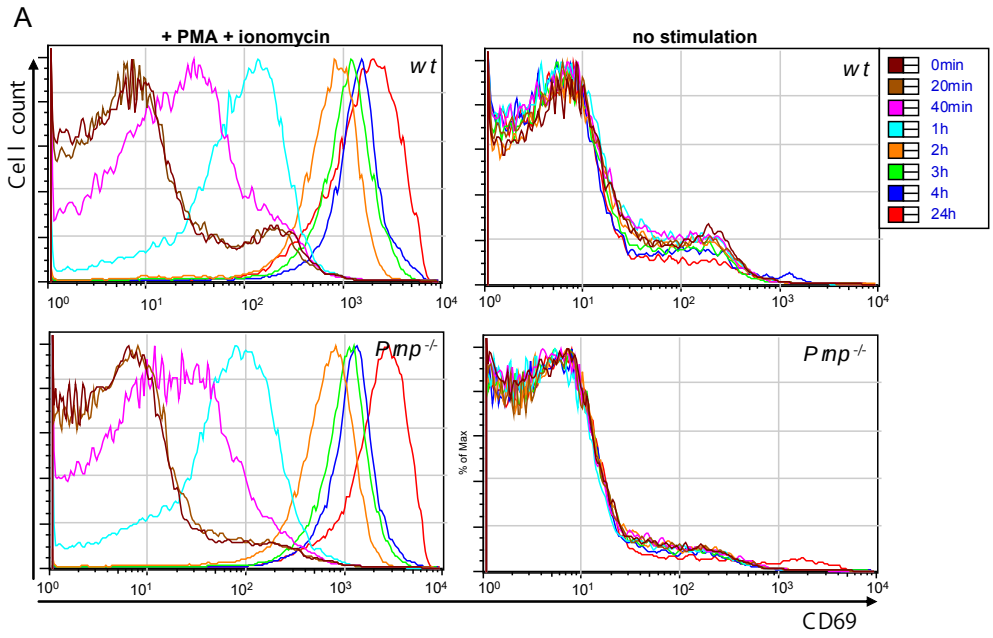
Few studies have been made available for characterization of various immune cell populations in terms of PrP^C expression. Liu and colleagues did a detailed analysis on PrP^C expression of various populations during B-cell development, with high focus on bone marrow cells, and found high expression on subpopulations of HSC (Liu et al. 2001). This last observation was later confirmed by observation that PrP^C is a marker for long term HSC in the bone marrow, which are $lin^-sca\text{-}1^+Endoglin^+$ (Zhang et al. 2006). The number of studies about PrP^C expression during T-cell development in murine thymus have also been limited, and have mainly suggested that PrP^C expression may be higher in DN and in CD4-SP populations (Kubosaki et al. 2001; Jouvin-Marche et al. 2006). These results were recently complemented by the observation that $CD4^+CD25^+Foxp3^+$ regulatory T-cells also express high levels of PrP^C (Isaacs et al. 2008). Finally it has also been shown that splenic follicular dendritic cells (FDCs) are PrP^{hi} (Brown et al. 1999b).

These are the most significant studies focusing on assessing the role PrP^C in lymphocyte development and homeostasis, and on characterizing its expression profiles in different murine lymphocytic populations.

4.2. Results

4.2.1. PrP^C expression increases in cells activated *in vitro*

The first step for assessing the role of PrP^C in lymphocyte activation was to assess *in vitro* the relation of these two parameters. For this purpose, splenocytes from adult wilt type (*wt*) mice were collected and to cultured *in vitro*, with or without 25ng/mL of PMA (phorbol 12-myristate 13-acetate) and 0,5µM of ionomycin, which should induce cell activation. The goal was to do a time course analysis of the membrane levels of CD69, a marker for early cell activation (Testi et al. 1989), and to evaluate if PrP^C expression would correlate with the cellular activation status.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+ PMA + ionomycin
no stimulation
wt
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Cell count
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CD69
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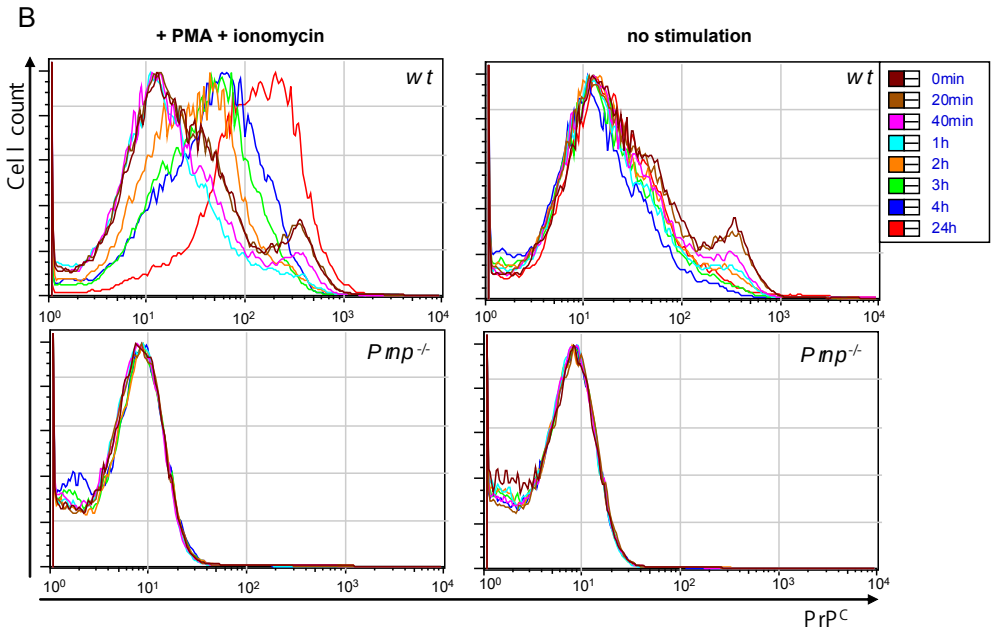
B
+ PMA + ionomycin
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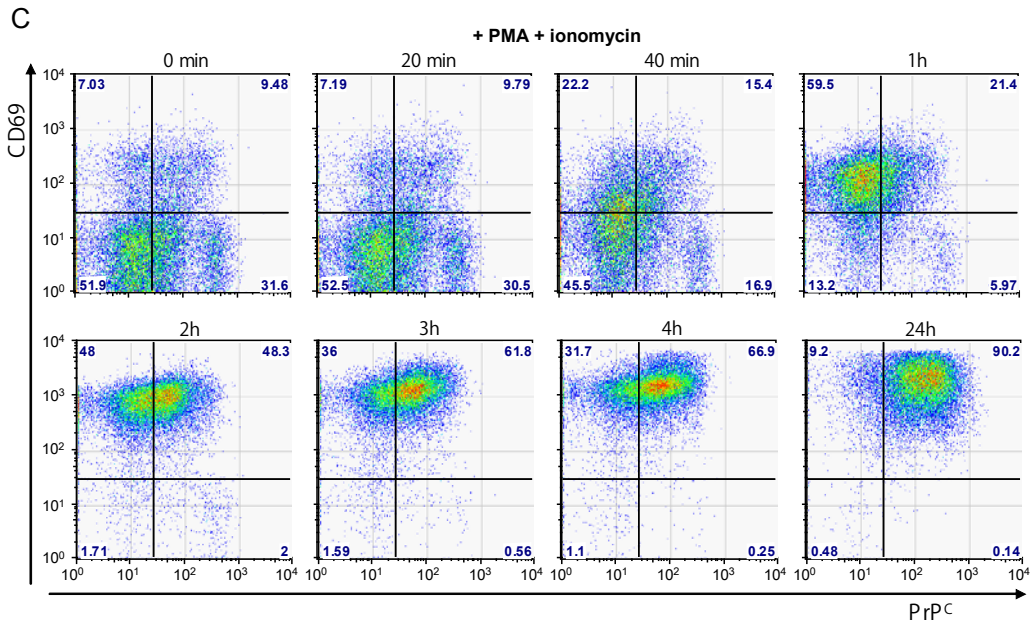
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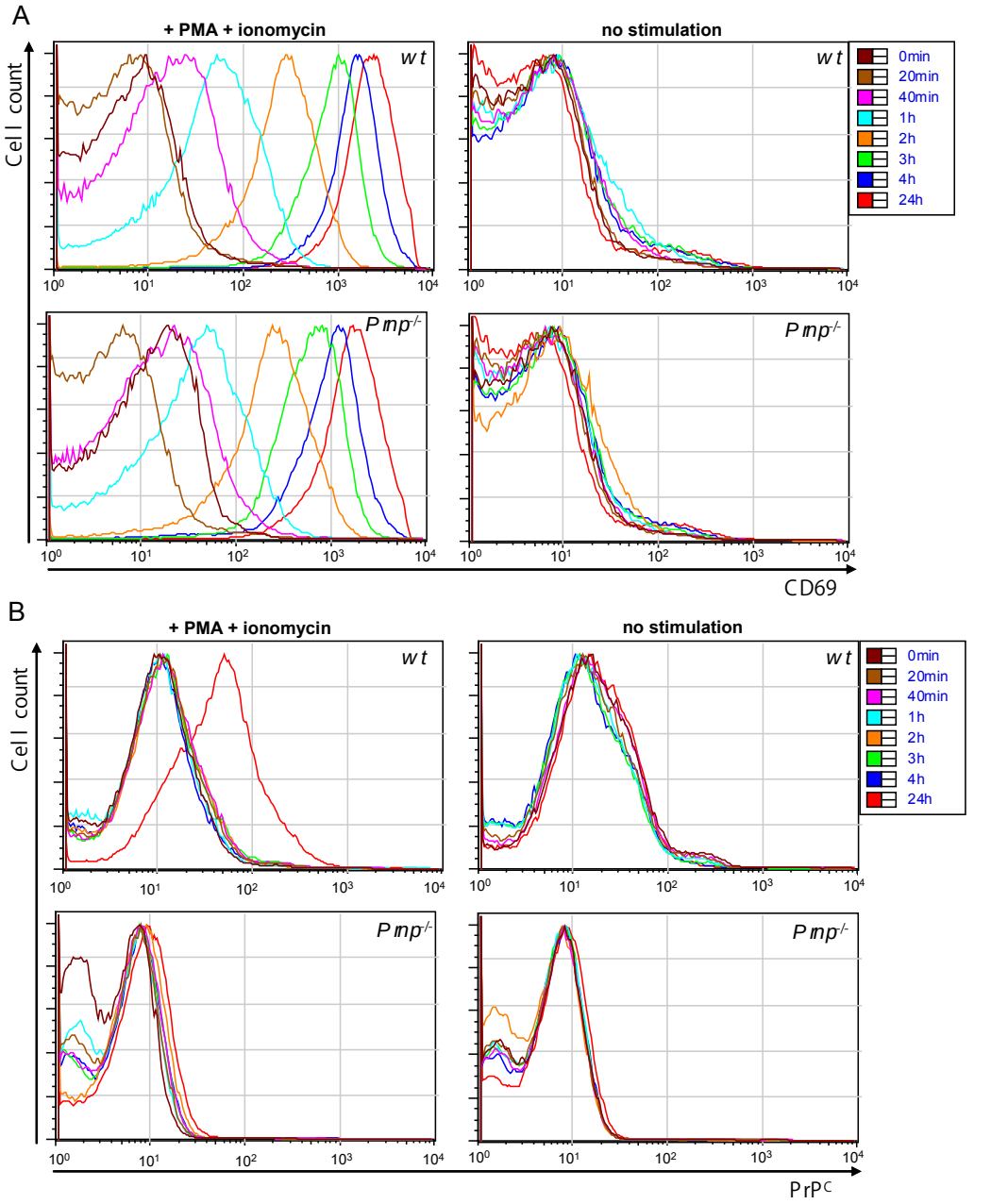
15.4
45.5
16.9
59.5
21.4
13.2
5.97
48
48.3
1.71
2
36
61.8
1.59
0.56
31.7
66.9
1.1
0.25
9.2
90.2
0.48
0.14

Figure 4.3: PrP^C and CD69 expression profiles on T-cells activated *in vitro*. Splenocytes from C57Bl6 mice (*wt*) and *Prnp*$^{-/-}$ mice were collected and cultured *in vitro* for different time periods, with or without 25ng/mL of PMA (phorbol 12-myristate 13-acetate) and 0,5µM of ionomycin. Populations were gated on TCRβ$^+$ T-cells. **A)** Quantification of surface CD69 levels at different time-points after cells were put in culture in presence of PMA and ionomycin (left panels) and in the absence of addition of stimulatory molecules (right panels). *Wt* cells were analysed (top panels) and *Prnp*$^{-/-}$ lymphocytes (low panels) were used as negative control. **B)** Quantification of surface PrP^C levels at different time-points after cells were put in culture in presence of PMA and ionomycin (left panels) and in the absence of addition of stimulatory molecules (right panels). *Wt* cells were analysed (top panels) and *Prnp*$^{-/-}$ lymphocytes (low panels) were used as negative control. **C)** Dot-plot of the *wt* T-cells cultured in presence of PMA and ionomycin, for each time-point of our experiment. Data illustrate the covariation of PrP^C and CD69 surface expressions.

Analysis of surface CD69 levels on TCRβ-gated T-cells suggest that the membrane levels of CD69 were highly elevated as early as 40 minutes after lymphocytes were put in culture (Figure 4.3 A), and that PrP^C levels followed shortly after, with high elevation already at 2h after cells were put in culture (Figure 4.3. B). Moreover, the kinetics of these two proteins correlated and their expression showed always a gradual increase, which lasted all the 24h of the experiment (Figure 4.3 A-C).

In contrast, on B-cells PrP^C appears to have a much different behaviour. While membrane levels of CD69 increase strongly on B220$^+$ splenocytes already at 40 minutes after cells were put in culture with the cell activators PMA and ionomycin (Figure 4.4 A), PrP^C up-regulation was only detected on these B-cells after 24h of culture (Figure 4.4 B-C).

Together, these results confirm observations reported by others on total human peripheral blood cells (Cashman et al. 1990) and on mouse splenocytes (Mabbott et al. 1997; Bainbridge and Walker 2005; Kubosaki et al. 2003), and add another level to their findings, showing for the first time that surface PrP^C can be up-regulated on T-cells as early as 2h after lymphocytes were put in culture, with a 20 to 80 minute delay of the protein surface up-regulation of CD69, which is one of the earliest activation markers known (Testi et al. 1994; Marzio et al. 1999). Moreover, it is to our knowledge the first time that such a study is performed on B-cells, illustrating the discrepancy of PrP^C up-regulation kinetics between T-cells and B-cells. Finally, such dramatic PrP^C up-regulation dynamics on T-cells suggests that PrP^C may be functionally linked to a pathway involving CD69.


A
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CD69
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15.6
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8.03
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3.97
79.9
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4.12
0.6
82.1
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0.18
87.1
11.9
1
0.082
32.3
67.4
0.21
0.048

Figure 4.4: PrP^C and CD69 expression profiles on B-cells activated *in vitro*. Splenocytes from C57Bl6 mice (*wt*) and *Prnp*$^{-/-}$ mice were collected and cultured *in vitro* for different time periods, with or without 25ng/mL of PMA (phorbol 12-myristate 13-acetate) and 0,5µM of ionomycin. Populations were gated on B220$^+$ B-cells. **A)** Quantification of surface CD69 levels at different time-points after cells were put in culture in presence of PMA and ionomycin (left panels) and in the absence of addition of stimulatory molecules (right panels). *Wt* cells were analysed (top panels) and *Prnp*$^{-/-}$ lymphocytes (low panels) were used as negative control. **B)** Quantification of surface PrP^C levels at different time-points after cells were put in culture in presence of PMA and ionomycin (left panels) and in the absence of addition of stimulatory molecules (right panels). *Wt* cells were analysed (top panels) and *Prnp*$^{-/-}$ lymphocytes (low panels) were used as negative control. **C)** Dot-plot of the *wt* B-cells cultured in presence of PMA and ionomycin, for each time-point of our experiment. Data illustrate the covariation of PrP^C and CD69 surface expressions.

4.2.2. PrP^C expression *in vivo* is increased in activated cells

Given the finding that PrP^C expression increases when cells are activated *in vitro*, I wanted to assess *in vivo* if the expression of PrP^C was increased in lymphocytes expressing activation markers. For this purpose splenocytes and lymph node cells expressing the activation marker CD69 were analysed by flow cytometry. As in agreement with the *in vitro* data, T-cells from lymph nodes (Figure 4.5 A-B), and from the spleen (Figure 4.5 C-D), as well as splenic B-cells (Figure 4.5 E-F), showed an increase in PrP^C at the cell surface in CD69$^+$ activated lymphocytes, comparing with non-activated CD69$^-$ ones. Interestingly, though in lymph nodes this augment was about 1log (Figure 4.5 B), in spleen this enhancement of PrP^C expression was much milder (Figure 4.5 D, F), suggesting that in spleen the levels of PrP^C are more tightly regulated, or that there may be mechanisms complementary to PrP^C function, with higher prominence in spleen.

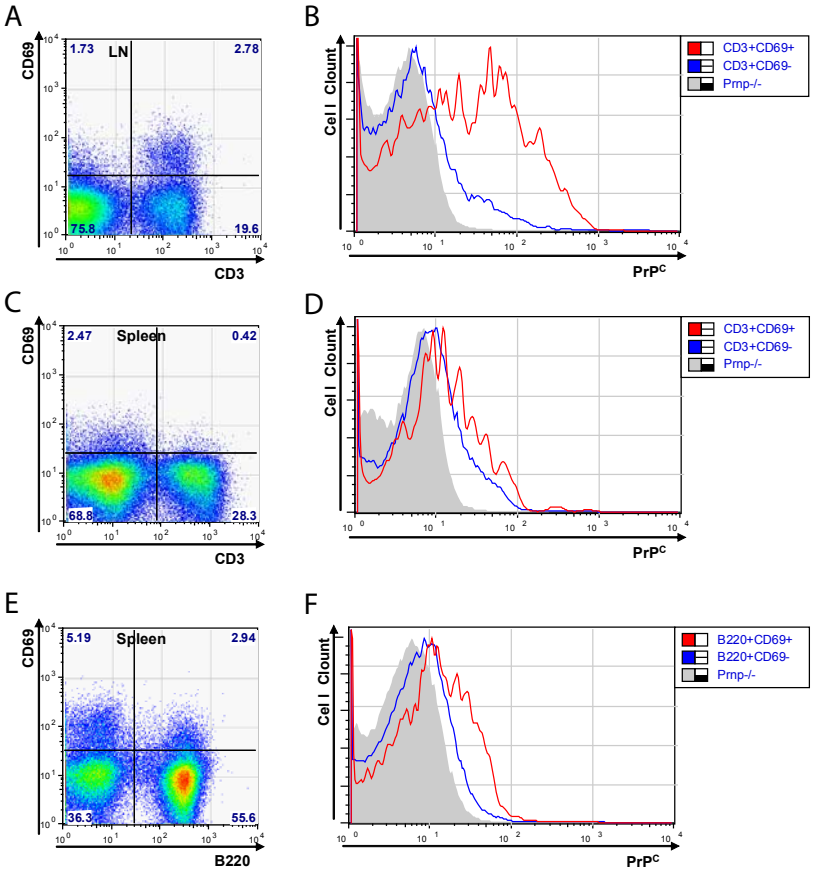

Figure 4.5: PrP^C expression is increased in activated cells: Lymph node and splenic cells from a *wt* mouse were collected and stained for CD3, a T-cell marker, B220, a B-cell marker, CD69, an early cell activation marker, and PrP^C. **A)** Dot-plot for lymph node cells showing activation of CD3$^+$ T-cells. **B)** Histogram for PrP^C expression of CD3$^+$ gated lymph node T-cells. CD69$^+$ population is shown in red and CD69$^-$ in blue. Grey refers to CD3$^+$ *Prnp*$^{-/-}$ cells. **C)** Dot-plot for splenocytes showing activation of CD3$^+$ T-cells. **D)** Histogram for PrP^C expression of CD3$^+$ gated splenic T-cells. CD69$^+$ population is shown in red and CD69$^-$ in blue. Grey refers to CD3$^+$ *Prnp*$^{-/-}$ cells. **E)** Dot-plot for splenocytes showing activation of B220$^+$ B-cells. **F)** Histogram for PrP^C expression of B220$^+$ gated splenic B-cells. CD69$^+$ population is shown in red and CD69$^-$ in blue. Grey refers to B220$^+$ *Prnp*$^{-/-}$ cells.

4.2.3. Characterization of surface PrP^C expression in various T-cell populations

Given this observation that PrP^C up-regulation in lymphocytes appears to be a marker for cell activation, it was then assessed if PrP^C expression correlated directly with the positive selection phases, and inversely with the phases where exclusion of auto-reactive cells occurred.

The T-cell development was assessed in thymocytes of C57Bl6 mice by comparing the expression levels of CD4 and CD8 (Figure 4.6. A), and by measuring the amount of CD44 in the DN-gated population (Figure 4.6 B). As described earlier, in the thymus the T-cell development phases start with a CD4$^-$CD8$^-$ (DN) CD44$^+$

phenotype, then DN $CD44^-$ (Figure 4.6 A-B), and it is during this DN stage that the TCRβ is rearranged and that positive selection for the pre-TCR occurs. After that, cells become $CD4^+CD8^+$ (DP) when positive and negative selection of TCRαβ takes place, and later become $CD4^+CD8^-$ or $CD4^-CD8^+$ (Figure 4.6 A).

In this study the average levels of PrP^C appeared to be higher in the DN phase, and to decrease in the remaining phases (Figure 4.6 C-D). These results are in agreement with previous publications that also showed a higher PrP^C expression in the DN population (Kubosaki et al. 2001), and in line with our rationale, they appear to correlate with the positive selection for a functional pre-TCR, which takes place in the same phase.

Given the observation that PrP^C expression was higher in DN $CD4^-CD8^-$ thymocytes I wanted to evaluate if this property is also visible in peripheral T-cells. For this purpose, the PrP^C levels in lymph nodal T-cells were assessed. As observed with the thymic T-cells, the higher PrP^C expressing population was the $CD3^+CD4^-CD8^-$. This subset may belong to the published $TCR\alpha\beta^+CD3^+CD4^-CD8^-$ double negative (DN) regulatory T-cell population (Zhang et al. 2000), which comprises 1-5% of the peripheral T-cells of human and mice (Thomson et al. 2006). The suggestion that a regulatory T-cell population was high in PrP^C expression prompted to assess the PrP^C levels profile of the classical $CD4^+CD25^+$ regulatory T-cell (T-reg) population (Sakaguchi et al. 1995). Comparison of the amounts of PrP^C in the cell membrane of $CD4^+CD25^+$ T-regs with the $CD4^+CD25^-$ population, suggested that this type of T-regs also expresses high amounts of PrP^C, which is in accordance with other publications (Isaacs et al. 2008). In line with the model proposed in this study, this observation can be explained by the fact that in the thymic selection process, this type of T-regs derives from the non eliminated T-cells that had a highest affinity for the Ag-complexed MHC class II (Jordan et al. 2001; Apostolou et al. 2002).

Taken together, in thymic T-cell development, the highest levels of PrP^C expression correspond to the positive selection phase for a functional TCRβ. Also, our data as well as other publications (Isaacs et al. 2008) suggest that PrP^C is present in high doses in populations of regulatory T-cells, which classically are selected among the T-cells that showed a higher reactivity for self antigens (Jordan et al. 2001; Apostolou et al. 2002). Again, this supports the suggestion that PrP^C is more expressed in cells whose endogenous activatory status appears to be higher.

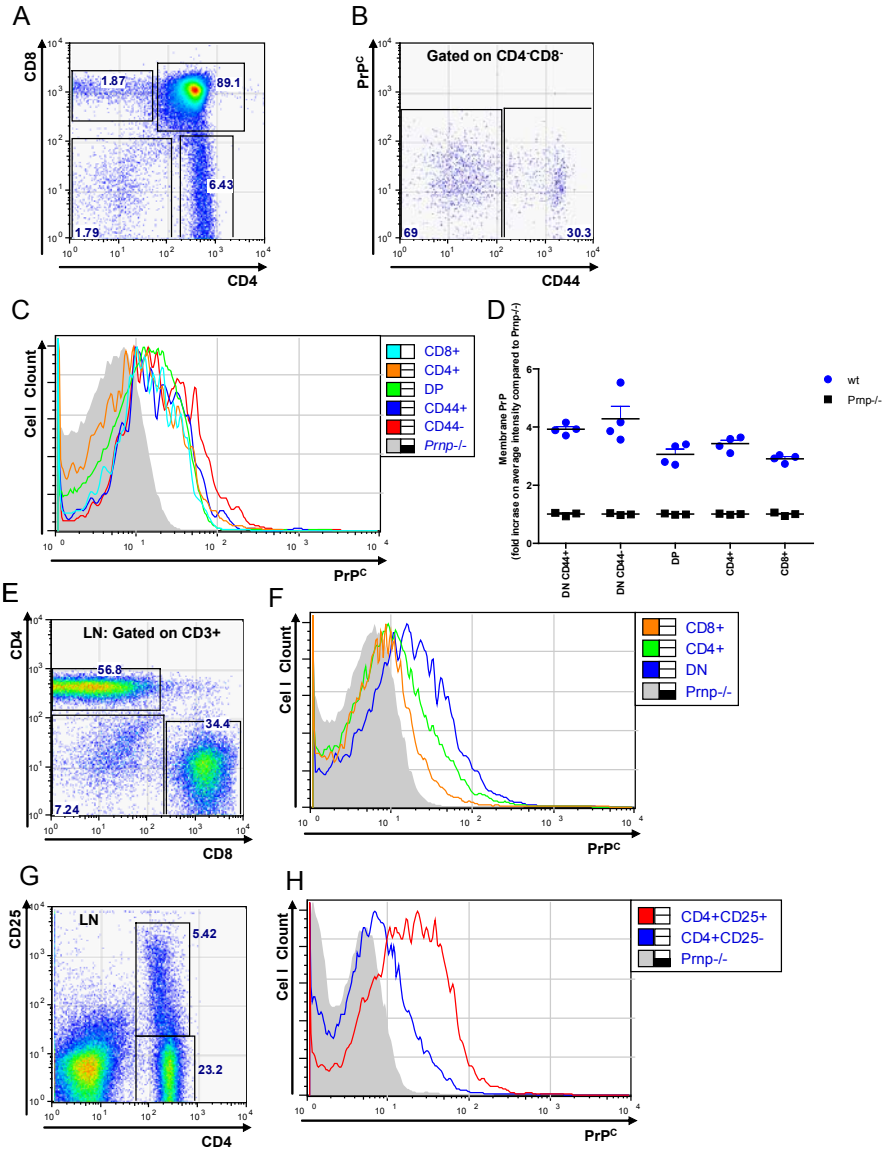

Figure 4.6: PrP^C expression in various T-cell populations: Lymphatic tissues from *wt* mice, and *Prnp*$^{-/-}$ as controls, were collected analysed by flow cytometry for various T-cell populations. **A)** Dot-plot for thymic lymphocytes showing expression of CD4 and CD8. **B)** Dot-plot for thymic CD4$^-$CD8$^-$ lymphocytes showing expression of CD44 and PrP^C. **C)** Histogram for PrP^C expression the Thymic populations shown in **A)** and **B)**. **D)** Quantification of the average PrP^C intensity of the populations shown in **C)** of 4 *wt* mice (blue) measured in fold increase over the average intensity for the *Prnp*$^{-/-}$ (black). Error bars represent the standard error of the mean. **E)** Dot-plot for CD3$^+$ gated lymph nodal lymphocytes with reference for the expression of CD4 and CD8. **F)** Histogram for PrP^C expression the Lymph nodal populations shown in **E)**. **G)** Dot-plot for lymph nodal lymphocytes with reference for the expression of CD4 and CD25. **H)** Histogram for PrP^C expression the Lymph nodal populations shown in **G)**.

4.2.4. Characterization of surface PrP^C expression in various B-cell populations

Like the T-cell development, B-cell development also has positive and negative selection processes. One of the checkpoints is at the end of Pro-B phase, where there is a selection for functional pre-BCR complexes. The other important step is at immature phase, where another positive and negative selection occurs. In order to evaluate if, like in T-cell development, an increase on PrP^C expression is correlated with states when cellular endogenous activity appears to be higher, the B-cell development was characterized in term of PrP^C expression. As represented in Figure 4.7 (A-B) for the bone-marrow of a C57Bl6 mouse, $B220^-CD19^-$ subset refers to a heterogeneous population of precursor cells for various lymphocytic lineages, including B-cell precursors. $B220^+CD19^-$ are the Pro-B-cells, that turn $B220^{lo}CD19^+IgM^-$ pre-B-cell, and $B220^{lo}CD19^+IgM^+$ are immature B-cells. Mature B-cells are generally represented as $B220^{hi}CD19^+$. Analysis of PrP^C expression in all these populations showed that PrP^C levels are much lower for bone-marrow B-cells, than for thymic T-cells (Figure 4.7 C-D). In addition, the subsets showing higher PrP^C levels are the $B220^-CD19^-$ heterogeneous population, in agreement with previous publications (Liu et al. 2001), and the Pro-B-cells that are in positive selection phase for a functional VDJ recombination in the heavy chain of the BCR (Figure 4.7 C-D), which agrees with the model the working hypothesis proposed in this study.

The other phase of B-cell receptor takes place in the spleen. There, transitional B-cells need to be positively selected for the BCR-Ag interaction, in order to proceed to mature follicular B-cells. However, the ones with lower interaction would turn into marginal zone cells (Cariappa et al. 2001). Therefore, in line with the observations that have been described in this work, it would be expectable a higher PrP^C expression in transitional B-cells, and low in MZ ones. However, the data suggested that the $B220^+CD21^{hi}CD23^{int}$ MZ B-cells express the highest levels, and $B220^+CD21^{lo}CD23^{lo}$ transitional, and $B220^+CD21^+CD23^{hi}$ follicular B-cells express the lower levers (Figure 4.7 E-H). But a careful evaluation of the histogram of Figure 4.7 F shows that transitional B-cells express similar levels of PrP^C as MZ, though the values of average intensity for this former population were lower than the ones for MZ, probably because Transitional B-cell appear to consist of an heterogeneous

population, with many PrP^C non-expressing B-cells, and with another population with higher levels for this protein (Figure 4.7 G-H). This apparent contradiction on PrP^C enhanced expression in MZ, and low affinity of these cells to the Ag during selection process (Cariappa et al. 2001) may be easily explained by the partial activated profile of the MZ B-cells (Oliver et al. 1997; Carvalho et al. 2001).

Taken together, in accordance with my observations with T-cells, in B-cells it also appears to exist a correlation between PrP^C expression profiles and the endogenous activatory status of the B-lymphocytes.

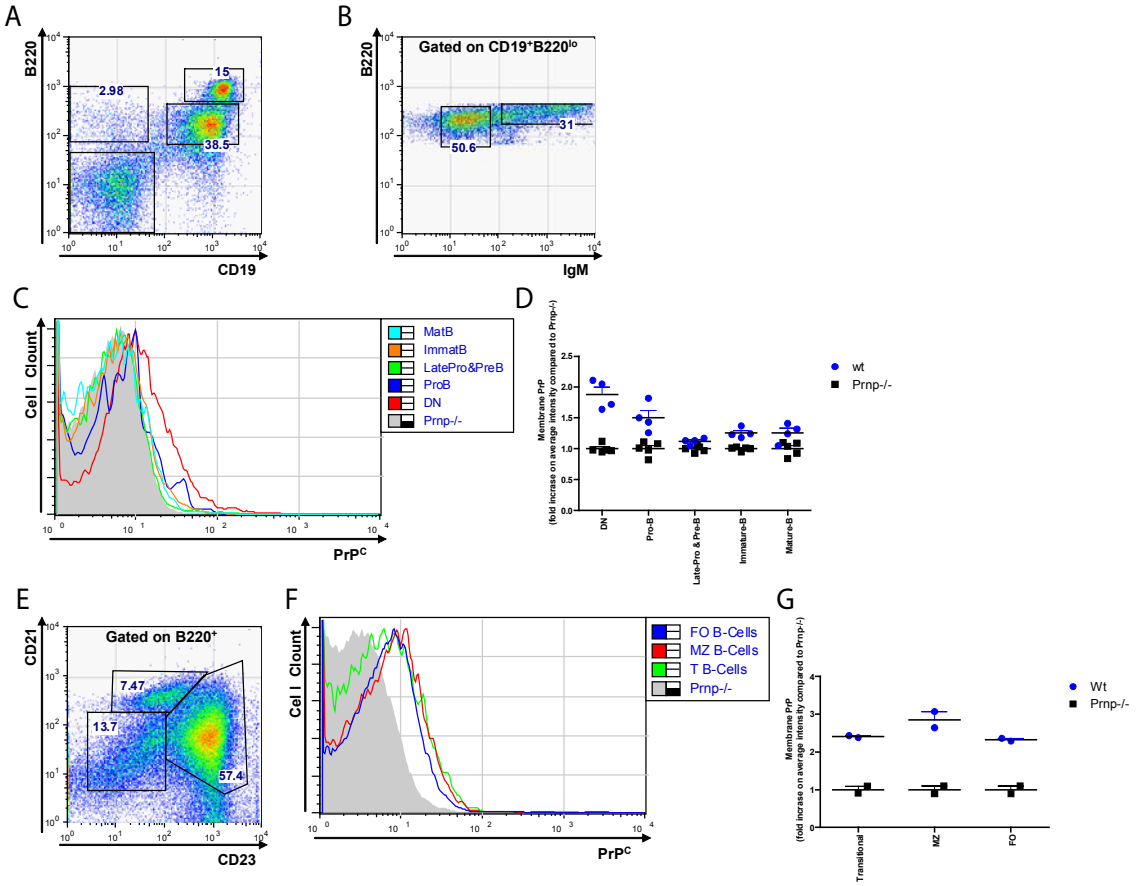

Figure 4.7: PrP^C expression in various B-cell populations: Lymphatic tissues from *wt* mice, and *Prnp*$^{-/-}$ as controls, were collected analysed by flow cytometry for various B-cell populations. **A)** Dot-plot for bone marrow lymphocytes illustrating various B-cell developmental phases, based on expression of B220 and CD19. **B)** Dot-plot for bone marrow $CD19^+B220^{lo}$ lymphocytes showing Pre and immature B-cell populations, based on expression of IgM and B220 and PrP^C. **C)** Histogram for PrP^C expression the bone marrow populations shown in **A)** and **B)**. **D)** Quantification of the average PrP^C intensity of the populations shown in **C)** of 4 *wt* mice (blue) measured in fold increase over the average intensity for the *Prnp*$^{-/-}$(black). Error bars represent the standard error of the mean. **E)** Dot-plot for B-cell final developmental steps in spleen, based on $B220^+$ gated cells with reference for the expression of CD21 and CD23. **F)** Histogram for PrP^C expression the splenic populations shown in **E)**. **G)** Quantification of the average PrP^C intensity of the populations shown in **F)** of 2 *wt* mice (blue) measured in fold increase over the average intensity for the *Prnp*$^{-/-}$(black). Error bars represent the standard error of the mean.

4.2.5. Study of the influence of altered expression of PrP^C in lymphocyte homeostasis

As discussed in the previous sections, PrP^C is differentially expressed during lymphocyte development, and it appears to be correlated with the activatory status of the cell. This observation, together with the model here described, suggests that PrP^C modulates the activation of lymphocytes. A corollary of this hypothesis is that artificial up-regulation of PrP^C in lymphocytes may increase their activatory condition and therefore induce the autoreactivity and consequently their elimination by negative selection, or their positive selection. Given this rationale, a short comparison of the lymphocytic homeostasis between wild type (wt), *Prnp*$^{-/-}$, and transgenic mice overexpressing PrP^C was performed.

The first step was to reproduce previously published data. Jouvin-Marche and colleagues made an exhaustive study of the T-cell development of *wt* mice, and two PrP^C overexpressing mouse lines: *Tg33*, which overexpressed PrP^C linked to the *lck* promoter (Raeber et al. 1999); and the transgenic *Tga20* line that overexpressed PrP^C earlier in lymphocyte development, because it used the ubiquitous *Prnp* promoter (Fischer et al. 1996). The conclusion of that study was that overexpression of PrP^C alters T-cell development in the thymus, namely increases the frequency of DN cells and decreases DP (Jouvin-Marche et al. 2006). In order to evaluate the reproducibility of those results, the T-cell development in *Tg33* and *Prnp*$^{-/-}$ normal littermates was assessed. Simultaneously, in order evaluate if the phenotype observed was age dependent, two litters of different ages were used: 12 week old, and a late age, 19 week old. According to Jouvin-Marche et al. (2006), the thymic phenotype of Tg33 appears from 10.5 weeks.

Analysis of T-cell development in *Tg33* and *Prnp*$^{-/-}$ mice showed no differences in the frequency of the various T-cell populations in the thymus of these mice (Figure 4.8 A-C). It appeared to be a trend for higher frequency of DN ($CD4^-CD8^-$) T-cells in aged mice (figure 4.8. A-B), but in any of the cases it was not detectable the previously reported effect of PrP^C overexpression in increasing the proportion of DN T-cells and decreasing the DP ($CD4^+CD8^+$) ones (Jouvin-Marche et al. 2006).

Following this study on the Thymus, an effect of PrP^C abnormal expression in B-cell development in bone marrow was investigated. For this, the late B-cell development of *Prnp*$^{-/-}$, wt, and the *CD19* promoter-linked PrP^C overexpressing *TgN431* transgenic

mouse line (Montrasio et al. 2001) was compared. Because both the transgenic lines were in a C57Bl6 and 129Sv mixed background, *wt* animals of these two backgrounds were included. Analysis of the frequencies of the various B-cell populations in bone marrow (Figure 4.8 D-E) showed a decrease of $IgM^{hi}IgD^{lo}$ immature B-cells, dependent on the PrP^C expression. In this experimental setup, absence of PrP^C increased the proportion on Immature B-cell in about 40%, and its overexpression decreased in other 40% the abundance of this $IgM^{hi}IgD^{lo}$ population (Figure 4.8 D-E). This observation is in agreement with the predictions that manipulation of doses of PrP^C expression may alter the activation status of the cell. In the Immature phase, lymphocytes are undergoing negative selection, in order to eliminate the ones that overreact to self antigens (Erikson et al. 1991; Hartley et al. 1991). Overexpression of PrP^C in high doses, may immediately dictate the elimination of these hyper-responsive cells. In contrast, hypo-responsive *Prnp*$^{-/-}$ immature B-cells would be arrested in the development in order do V-J rearrangements in the light chain of the immunoglobulin.

Other cell populations in the periphery were also analysed, namely the $CD3^+CD4^-CD8^-$ population that was shown previously to be a high PrP^C-expressing subset (Figure 4.8 F-G). These cells have been referred to include the DN regulatory T-cells (Zhang et al. 2000; Ford et al. 2006), which were suggested to comprise 1-5% of the peripheral T-cells of human and mice (Thomson et al. 2006). It appeared that in lymph nodes, the abundance of these cells may be directly modulated by the amount of PrP^C expressed, with a very high frequency in *Tga20* mice. The particularity of the *Tga20* phenotype has been suggested by others to be also influenced by the insertion site of the *Prnp* genes (Zabel et al. 2008). This is in agreement with the observation that regulatory T-cells are hipper-responsive cells, which are classically selected among the T-cells with higher reactivity for self antigens (Jordan et al. 2001; Apostolou et al. 2002).

Taken together, data here suggests that the published effect of PrP^C overexpression on T-cell development (Jouvin-Marche et al. 2006) is not dependent on PrP^C expression, given the fact that *Prnp*$^{-/-}$, which was not assessed in the original publication, and the PrP^C overexpressing *Tg33* mouse, had no notorious differences in T-cell development in thymus. In addition, data suggests that PrP^C expression may influence the positive and negative BCR selection events on immature B-cells in the bone marrow. Finally, it suggests the existence of an expansion of the regulatory T-

cell compartment in transgenic mice expressing high levels of PrP^C. This observed inverse correlation between dose of PrP^C and percentage of immature B-cells, and direct correlation with the abundance of regulatory T-cells is in agreement with the results already discussed here and with the working hypothesis that PrP^C modulates the activatory status of a cell.

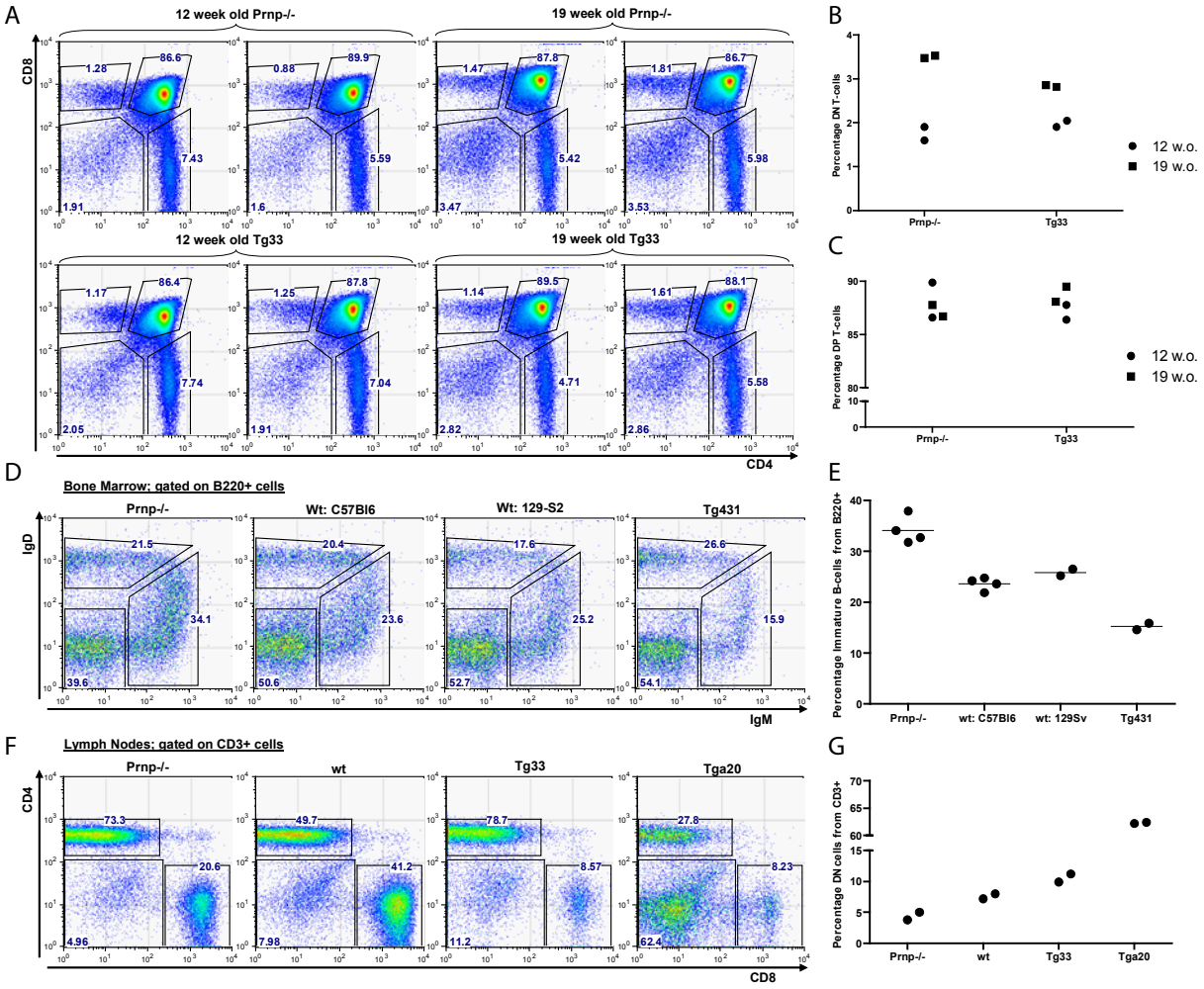

Figure 4.8: Influence of altered expression of PrP^C in lymphocyte homeostasis. A) Dot-plot illustrating thymic T-cell developmental populations from *Prnp*$^{-/-}$ mice (top) and PrP^C overexpression *Tg33* (bottom), with 12 (left) or 19 (right) weeks of age. **B)** and **C)** Quantification of the percentage of $CD4^-CD8^-$ DN **(B)** and $CD4^+CD8^+$ DP **(C)** populations from the dot-plots form **A)**. **D)** Dot-plot illustrating bone marrow B-cell developmental populations from *Prnp*$^{-/-}$ (first panel), C57Bl6 (second panel), 129Sv (third panel) and PrP^C overexpressing *TgN431* mice (fourth panel). **E)** Quantification of the percentage of $IgM^{hi}IgD^{lo}$ immature B-cell population from the results represented by the dot-plots in **D)**. **F)** Dot-plot illustrating lymph nodal T-cell populations from *Prnp*$^{-/-}$ (first panel), *wt* (second panel), and PrP^C overexpressing *Tg33* (third panel) and *Tga20* (fourth panel). **E)** Quantification of the percentage of $CD3^+CD4^-CD8^-$ T-cells population from the results represented by the dot-plots in **F)**.

4.2.6. Role of PrP^C in TCR signalling

Given the data described above, suggesting that PrP^C expression alters the lymphocytic homeostasis, I decided to evaluate the role of PrP^C in modulating the intensity of the signals that reach a lymphocyte through its antigen receptor. The working hypothesis was that overexpression of PrP^C would decrease the lymphocytic activation threshold, and therefore promote the positive selection of lymphocytes with lower expression of the costimulatory molecules that participate in the antigen receptor signalling. A result of this model would be that the positively selected T-cells that proliferating in the periphery would have a lower expression of CD3, in order to weaken the signal provided to the cell, through interaction of MHC with the Ag (Gil et al. 2005; Kuhns et al. 2006).

In order to test this hypothesis, it was first assessed by flow cytometry the expression of surface CD3 in T-cells in lymph nodes, in mouse lines overexpressing PrP^C, *Tg33* and *Tga20*, and in *wt* and $Prnp^{-/-}$ mice. A first approach confirmed that mature T-cells of mice overexpressing PrP^C had lower quantity of the costimulatory CD3 molecule at the surface, comparing with *wt* and $Prnp^{-/-}$ mice (Figure 4.9 A-B). The working hypothesis would also predict that absence of PrP^C would select for lymphocytes with higher expression of CD3, however, there were detected no differences between $Prnp^{-/-}$ and *wt* (Figure 4.9 A-B). This could indicate that T-cells are more sensitive to overexpression of PrP^C, than to the absence of it.

These results were later confirmed when it were compared the levels of CD3 expression in T-cells from the spleen of $Prnp^{-/-}$ mice and PrP^C overexpressing *Tg33* normal littermates. In spite of the high variability observed in the $Prnp^{-/-}$ sample, in this paradigm it was again observed the trend of detecting lower quantity of CD3 at the membrane in mice overexpressing PrP^C (Figure 4.9 C-D). Furthermore, it appeared that in these transgenic conditions, lymphocytes in older animals were more susceptible to the effect of overexpressing PrP^C, and showed a more pronounced trend for down-regulation CD3 (Figure 4.9 C-D).

These evidences for a role of PrP^C in modulating the cell activation status, prompted for a more crucial experiment. I decided to evaluate if overexpression of PrP^C in thymocytes would partially revert the absence of MHC class II, in the positive selection process. For this, the Aa^0/Aa^0 mice were used. These animals completely lacked MHC class II (Kontgen et al. 1993), and were crossed with PrP^C

overexpressing *Tg33* mice. Knocking out MHC class II is not sufficient to abolish CD4 T-cell development, because there are cases of low affinity interactions of CD4 molecule with MHC class I, which may allow the positive selection and the maintenance of a small population of CD4+ T-cells (Kontgen et al. 1993; Chan et al. 1998). The working hypothesis was that PrP^C up-regulation in mice devoid of MHC class II, would result in a decrease of the activation threshold, and therefore allow the maintenance of a slightly larger population of CD4 T-cells.

Analysis of the lymph nodes of *Prnp*$^{+/-}$ *Aa*^{0}/*Aa*^{0} mice showed a large presence of CD8 T-cells, comparing with CD4 T-cells (Figure 4.9 E-F). However, overexpression of PrP^C resulted in the maintenance of an about 10% larger population of CD4 T-cells (Figure 4.9 E-F). Though this increase was not very pronounced, it provides another piece of evidence for a role of PrP^C in modulating cell activation.

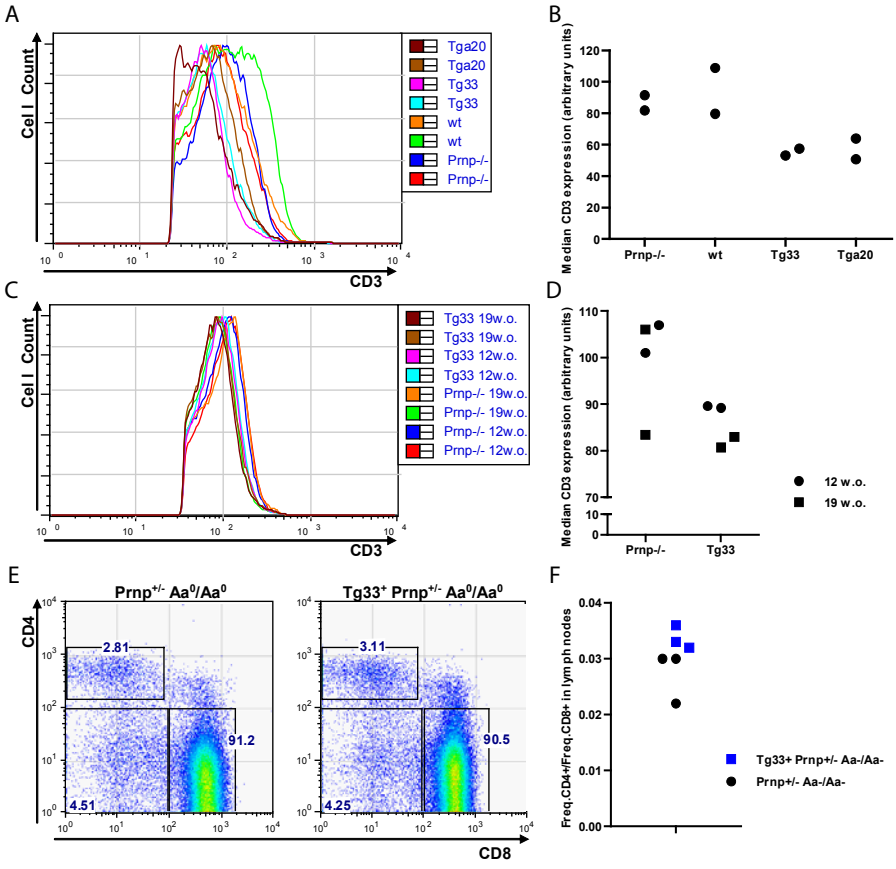

Figure 4.9: PrP^C influences TCR signalling. A) CD3+ gated histogram for CD3 expression the lymph node T-cell populations of *Prnp*$^{-/-}$, *wt*, and PrP^C overexpressing *Tg33* and *Tga20* mice. **B)** Quantification of the median CD3 intensity of the populations shown in **A)**. **C)** CD3+ gated histogram for CD3 expression the splenic T-cell populations of *Prnp*$^{-/-}$ and PrP^C overexpressing Tg33 normal littermates of 12 and 19 weeks of age. **D)** Quantification of the median CD3 intensity of the populations shown in **C)**. **E)** Representative dot-plots for CD4 and CD8 expression on $CD3^+$ gated lymph node cells of MHC class II deficient *Prnp*$^{+/-}$ *Aa*^{0}/*Aa*^{0} mice and PrP^C overexpressing MHC class II deficient Tg33 *Prnp*$^{+/-}$ *Aa*^{0}/*Aa*^{0} normal littermates. **F)** Quantification of the $CD4^+$/$CD8^+$ ratio from the dot-plots shown in **E)**, with n=3.

4.3. Discussion

The goal for this set of experiments was to test the assumption that overexpression of PrP^C would modulate cell activation, and that a continuous non-physiological maintenance of an activatory status of many cells could lead to toxicity and degeneration of a certain system. In fact many functions, properties and interacting partners have been already appointed to PrP^C, which could all be explained by this assumption (Aguzzi et al. 2008a; Hu et al. 2008; Zomosa-Signoret et al. 2008; Mazzoni et al. 2005), however there was the need to confirm this paradigm, given the central role it played in the general model proposed here, for the role of PrP^C in neurotoxicity. In this chapter it was shown that PrP^C is quickly up-regulated upon T-cell activation, in a time-frame faster that what has been reported. It also provided for the first time evidence for PrP^C being up-regulated upon B-cell activation, but in a much less pronounced fashion. These results were confirmed *in vivo* by showing that B and T-cell in the periphery, expressing the activation marker CD69, also expressed higher levels of PrP^C. Furthermore, data also indicated that in B and T-cell development, the higher PrP^C expresser cells are the ones that are facing positive selection and therefore have a higher activatory status, like the Pro-B-cells in bone marrow and the DN T-cells in thymus. And this pattern was also detectable in other population with a higher activatory status, like regulatory T-cells and marginal zone B-cells.

But more than providing evidence for a correlation between abundance of PrP^C and the activatory status of a cell, this work aimed to show that altered PrP^C expression interferes with the lymphocyte developmental stages where positive or negative selection are involved. Data suggested that this does not occur during the various thymic T-cell developmental stages, therefore contradicting the conclusions achieved by others (Jouvin-Marche et al. 2006). In this respect, the results presented here indicated that T-cell development in PrP^C overexpressing *Tg33* mice appears to be similar to the one of *Prnp*$^{-/-}$ mice, and in the studies referred (Jouvin-Marche et al. 2006), this knockout mouse line was not studied. I propose that the absence in previous studies of data relative to the *Prnp*$^{-/-}$ line (Jouvin-Marche et al. 2006) may have been the main reason for this contradiction. But despite of the absence of evidence for a role of PrP^C in modulating T-cell development in thymus, data here

indicated that PrP^C expression may modulate other processes in lymphocyte development and homeostasis, as the abundance of regulatory T-cells and the amount of immature B-cells at the bone marrow. This last population had a lower frequency in transgenic mice expressing high doses of PrP^C at the cell membrane, and was elevated in *Prnp*$^{-/-}$ animals, which can result from the fact that overexpression of PrP^C may have induced a higher activatory status on the cell, and consequent deletion due to over-reactivity against self antigens. Also, a lower activatory status induced by deletion of PrP^C should result in an increase of anergy of immature B-cells, and consequent arrest at this stage, for BCR recombination.

Such an effect of PrP^C in the activatory status of a lymphocyte would imply that the T-cells in the periphery, which would transgenically express high doses of PrP^C, would have to decrease the expression of the proteins that positively participate in the TCR signalling. And in fact this was observed in PrP^C overexpressing *Tga20* and *Tg33* mice. Though with a small sample size, it was shown here that in two independent experimental setups, there is a trend for a decrease of the expression of membrane CD3 in T-cells from mice with high doses of PrP^C, both in lymph nodes and in spleen. But the most convincing result for demonstrating that PrP^C is a modulator of the activatory status of a cell was the observation that PrP^C overexpression could partially revert the CD4 T-cell selection arrest of mice devoid of MHC class II. This is the first time that it was shown that overexpression of PrP^C can compensate the deficiency of MHC molecule and therefore provides strong evidence for a participation of PrP^C in the TCR signalling pathway, or in a complementary cell activation cascade.

There are however two main arguments that main weaken the certainty of the data presented here. One is the low sample size used in some of the experiments, the other is the fact that the background differences of the mice used and the transgene insertion site on the transgenic lines advise for caution in interpreting the results. On the other hand, this work provided a large set of experiments, using in total two *wt* and six transgenic mouse lines, and although the results obtained sufficed to disprove previous research works, they never contradicted the main working hypothesis of this work, but rather supported it. Furthermore, many of the observations presented here, were supported by previous reports. For these reasons, the most parsimonic explanation for the data presented here and for the

observations done in other reports is that PrP^C modulates the activatory status in certain lymphocyte populations.

4.4. Outlook

Despite the conclusion described here results from the observation of a large set of experiments performed in many different paradigms and using a total of 8 mouse lines, this study still has some weaknesses that can be easily overcome. One of the actions to be taken is to increase the sample size of these experiments. Another important experiment would be to graft lymphocytes from the various mouse lines used, into a single recipient mouse, and to evaluate the influence of PrP^C expression would favour the selection of certain lymphocyte populations.

The “Holly Grail” of prionology is to uncover the main physiological function of PrP^C. For this, one of the approaches is to assess the molecular pathways where PrP^C is integrated. In this study I described a phenotype of mice with altered PrP^C expression, and showed that CD3 is down-regulated in transgenic mice overexpressing PrP^C. Thus, it is important to evaluate the position of PrP^C in the TCR/CD3 signalling pathway. PrP^C is located in the lipid rafts, and therefore it is possible that it participates in the immunological synapse. On the other hand, PrP^C may function internally, upon its internalization. These possibilities urge to be clarified.

Another important experiment is to evaluate if PrP^C mutations that result in toxicity act on the same pathway as PrP^C overexpression. This could be done either using the MHC class II deletion model, or by assessing if CD3 and other TCR costimulatory molecules, are also down-regulated in mice bearing toxic PrP molecules. As a follow-up, it would be important to investigate if PrP^{Sc} would also act in the same pathway. The integration of these 3 PrP features (PrP^{Sc}, PrP toxic mutants, and PrP^C overexpression at very high doses) in a single pathway is another of the main assumptions of our model, and for many years has been a central question in the field.

Many other further studies can be done using this system, however, I consider that the focus of future work should be in: i) Identifying the main molecular pathways

influenced by PrP^C expression; ii) Evaluating if PrP^{Sc}, PrP^C toxic mutants, and PrP^C abnormal overexpression share the same toxic pathways.

4.5. Materials and methods

4.5.1. Mice used

Tga20 (Fischer et al. 1996), Tg33 (Raeber et al. 1999), and TgN431 (Montrasio et al. 2001), are PrP^C overexpressing transgenic mice, with the *Prnp* gene linked to the *Prnp*, *Lck*, and *CD19* promoter, respectively. These mice, together with $Prnp^{-/-}$ mice (Büeler et al. 1992) are all in a background mixture of C57Bl6 (Harlan Laboratories) and 129Sv (Charles River). Aa^0/Aa^0 mice (Kontgen et al. 1993) were in C57Bl6 background, and were crossed with Tg33. F_2 $Tg33^+Prnp^{+/-}$ Aa^0/Aa^0 were used. If not described, *wt* designation always referred to C57Bl6 strain. Unless stated otherwise, mice were 12 week old.

4.5.2. Flow cytometry

Mouse organs were collected, and cell suspensions were kept on ice, in PBS 2% FCS.

Antibodies used for stainings were PerCP- and PE- labelled CD3 and CD4, PE- and APC- CD8, PE- CD69, FITC- and PerCP- B220, FITC- CD44, PE- CD25, FITC- CD21, PE- CD23, PE- CD43, PE- CD19, FITC- and PE- IgM, PE- IgD, and FITC- IAb, all were obtained from BD Biosciences. Anti PrP POM2 antibody (Polymenidou et al. 2008) was labelled with Cy5.

Data was analysed using FlowJo software.

4.5.3. *in vitro* lymphocyte culture

Splenocytes were obtained from C57Bl6 and *Prnp*$^{-/-}$ mice. Cells were cultured with RPMI, 10%FCS, 1mM NaPiruvate, and 0.05mM 2-ME, in presence or absence of 25ng/ml PMA (phorbol 12-myristate 13-acetate) and 0.5uM ionomycin (ionophore). Cells were harvested at the various time-points and kept on ice. All samples were stained and analysed at the same time.

5. Final considerations

The term “Prion” has more than 25 years old (Prusiner 1982), and the fundaments of the protein-only hypothesis are from the 60’s (Griffith 1967). But in spite of the big effort put in prion research in all these years, the main fundamental questions are still unanswered: The physiological function of PrP^C remains elusive; the published data about interacting partners of PrP^C is largely contradictory; the mechanisms of PrP-dependent toxicity are still an enigma; and there is even no certainty about the number of mechanisms on how an altered PrP^C molecule can result in toxicity.

The main aim for this work was to draw a model that would conciliate all the information available on PrP^C function and toxicity. This was obviously a very ambitious goal, but nevertheless I elaborated a model on the function of PrP^C and on the mechanisms of PrP-dependent toxicity induced by PrP^{Sc}, PrP^C mutants, and probably abnormal PrP^C overexpression (Westaway et al. 1994b). I believe that the model presented in this dissertation is the one that congregates more data and has the higher parsimony.

The step was to investigate the mechanism that I proposed to the driving one for regulating the function of PrP^C. I rationalised that α-cleavage of PrP^C was the most significant process by which PrP could be regulated, and the main factor that would explain the PrP-dependent toxicity. Therefore, I dedicated my efforts in understanding the way α-cleavage is regulated. My results provided a characterization of the PrP^C domains and factors involved in modulating this cleavage, which illustrated for the first time the exceptional features of this proteolytic process. α-cleavage appears to be largely sequence independent, charge independent, hydrophobicity independent, of the region of cleavage site. These characteristics are not common in the literature, but may find a certain degree of parallelism in the cleavage plasticity of γ-secretase.

Following the study on the characterization of the main player in the conceptual model proposed here, I decided to validate the main assumption on this system. I assumed that PrP^C internalization would lead to cell activation, and that a miss-regulation of this process could be the basis of PrP-dependent toxicity. For this, I chose to use the immune system as a biological model for cell activation, mainly because lymphocytes undergo positive and negative selection during its

development, and therefore may be used to test situations of cell hypo- and hyper-reactivity. Data indicated that PrP^C is up-regulated upon lymphocyte activation and that the populations with more elevated levels of PrP^C expression are generally the ones that are activated or that have a higher reactivity or activatory status. It also suggested that altered expression of PrP^C results in an alteration of the lymphocytic homeostasis, favouring the elimination of lymphocytes undergoing negative selection for hyper-reactivity, in case of overexpression of PrP^C, and the inverse if PrP^C was knocked-out. Finally, data provided evidence for an influence of PrP^C in the TCR signalling pathway: positively selected lymphocytes overexpressing PrP^C had a reduction in CD3 expression, and additionally, high levels of PrP^C partially reverted the phenotype of mice devoid of MHC class II, by enhancing the positive selection of CD4 T-cells.

Globally this work provides a unified model of PrP^C function and of the PrP-dependent mechanisms of toxicity. It also characterizes the α-cleavage of PrP^C, which is the main driving force of the model here proposed, and provides data supporting the main assumption of the model, which is that PrP^C expression modulates cell activation.

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Curriculum Vitae

IDENTITY:

BIRTH	1980-12-08 - Portugal
NATIONALITY	Portuguese

ACADEMIC DEGREES:

- 2003 - Degree in Biology at University of Évora – Portugal

Graduation Thesis: "Modeling Epidemiology and Evolution of Influenza A Virus"

AWARDS:

- 2002 - Merit scholarship for best student of Biology of University of Évora – Portugal – in the year 2000/01.

FELLOWSHIPS:

- 2004-2008 – PhD Fellowship from Portuguese Foundation for Science and Technology
- 2003-2004 – Gulbenkian Foundation Fellowship for Gulbenkian PhD Program in Biomedicine
- 2003 – PRODEP Fellowship awarded via University of Évora - Portugal, for training at Gulbenkian Institute of Science - Portugal

PUBLICATIONS:

- In preparation: **José B. Oliveira-Martins**, Sei-ichi Yusa, Anna Maria Calella, Claire Bridel, Frank Baumann and Adriano Aguzzi: Alpha-Cleavage of the Prion Protein tolerates sequence degeneration and is controlled by length of domain 106-119.
- 2006 - Dinis Gökaydin, **José B. Oliveira-Martins**, Isabel Gordo and M. Gabriela M. Gomes: The reinfection threshold regulates pathogen diversity: the case of Influenza. J. R. *Soc. Interface*

REFEREE REPORTS:

- Refereed for the journal "Biology Letters" from the Royal Society of London.

WORKING EXPERIENCE:

- Since Jan 2005: Researcher at the Institute for Neuropathology of the University Hospital of Zurich – Switzerland, supervised by Adriano Aguzzi.

  Project Title: Candidate approach study of the main areas in PrP^{C} Central Domain involved in regulation of PrP^{C} cleavage at 109KH110.

  Project Title: Study the physiological function of PrP^{C} in Lymphocyte homeostasis

- May 2004 - Dec 2004: Researcher at the Lymphocyte Physiology Group of Instituto Gulbenkian de Ciência – Portugal, supervised by Jocelyne Demengeot.

  Project Title: Live imaging of lymphocyte motility by intravital microscopy

- Sep 2002 - Sep 2003: Researcher at the Theoretical Epidemiology Group of Instituto Gulbenkian de Ciência – Portugal, supervised by M Gabriela M Gomes.

  Project Title: Mathematical Modeling of Epidemiology and Evolution of Influenza A Virus

- Apr 2001 - Oct 2001: Researcher at the Arachnology Group of University of Évora – Portugal, supervised by Carola Meierrose

  Project Title: Biodiversity studies in fallows of varied duration in the area of Castro Verde (Southern Alentejo, Portugal) – Spider fauna